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AMERICAN MALACOLOGICAL BULLETIN 19 • 1/2 • 2004

Morphology and phylogenetic relationships of genera of North American Sphaeriidae (Bivalvia, Veneroida)

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Abstract: Recent phylogenetic studies of the Sphaeriidae have produced conflicting results between morphological and molecular data sets for two major topological elements, *i.e.*, monophyly of *Pisidium* and of asynchronous brooders (*Sphaerium* and *Musculium* taxa). Gene trees indicate a paraphyletic *Pisidium* while morphological trees suggest it is a derived monophyletic clade. Molecular analyses recover a derived monophyletic clade of asynchronous brooders suggesting the evolutionary elaboration of brooding character complexity from synchronous to sequential brooding. Conversely, morphological analyses indicate a sister relationship among *Musculium* and *Pisidium* taxa and propose that reduced *Pisidium* characters are derived from larger plesiomorphic *Sphaerium* taxa. To test these competing hypotheses of sphaeriid relationships, the morphology of North American sphaeriid taxa was studied, and major anatomical and developmental features that have been considered fundamental by previous workers were coded. Parsimony analyses with and without outgroup rooting showed that *Sphaerium* and *Musculium* taxa form a monophyletic group, congruent with previous gene trees but not with morphological studies. The conflict between molecular and morphological trees for the monophyly of *Pisidium*, however, remains unsettled. At present, informative morphological characters appear insufficient to flesh out phylogenetic relationships among infra-generic sphaeriid taxa.

Key words: Sphaeriinae, freshwater clams, phylogeny, brooding character evolution

Conflicts between the trees inferred from morphological and molecular characters are not rare in phylogenetic literatures (see Baker *et al.* 1998). Gene trees may not reflect species phylogeny when the gene(s) investigated have experienced confounding evolutionary processes such as lineage sorting, horizontal transfer, introgression, and ancestral polymorphism (Doyle 1992, Brower *et al.* 1996). In many cases, however, levels of incongruence between morphological and molecular topologies were artificially inflated due mainly to the lack of close examination of either or both types of data (Hillis and Wiens 2000). Recently, independent analyses of morphological (Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002) and molecular (Cooley and Ó Foighil 2000, Lee and Ó Foighil 2003) data sets resulted in incompatible sphaeriid phylogenies. The aim of this study was to test these competing hypotheses of sphaeriid relationships by reevaluating morphology-based phylogeny.

The Sphaeriidae (fingernail/pea/pill/nut clams) are known from the Cretaceous (Keen and Dance 1969), representing one of the major molluscan freshwater radiations (McMahon 1991) and presently have a cosmopolitan distribution in virtually all kinds of freshwater habitats (Clarke 1973, Burch 1975, Kuiper 1983). Although sphaeriid clams are the smallest bivalves in freshwater, they often constitute a large proportion of the benthic fauna in streams and ponds (Avolizi 1976, Eckblad *et al.* 1977) and are important components in energy and nutrient cycling (Alimov 1970, Hornbach *et al.* 1984, Holopainen and Hanski 1986, Lopez and Holopainen 1987, Way 1988). Sphaeriids exhibit a remarkable degree of genome amplification (up to 13n) (Park 1992, Barsiene *et al.* 1996, Burch *et al.* 1998, Lee 1999) and a number of North American species may share ancestral genome duplication events that predate their cladogenesis (Lee and Ó Foighil 2002).

Sphaeriid taxonomy

Convincing marine outgroups for the Sphaeriidae are presently lacking. The long-assumed monophyly of the superfamily Corbiculoidea comprising the Sphaeriidae and Corbiculidae (Newell 1965, Keen and Casey 1969, Taylor *et al.* 1973, Boss 1982, Morton 1996) has been rejected by both morphological (Dreher-Mansur and Meier-Brook 2000) and molecular (Park and Ó Foighil 2000, Giribet and Wheeler 2002) studies. Similar brooding characters observed in sphaeriids and freshwater corbiculids are thought to represent convergent adaptations to freshwater habitats rather than shared-derived homologies (Park and Ó Foighil 2000).

Five sphaeriid genera (*Byssanodonta* d'Orbigny, 1846, *Eupera* Bourguignat, 1854, *Musculium* Link, 1807, *Pisidium* Pfeiffer, 1821, *Sphaerium* Scopoli, 1777) have been widely recognized based on shell and soft-part morphology and reproductive/developmental characteristics (Odhner 1921,

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1922, Baker 1927, Heard 1965b, Burch 1975, Kuiper 1983, Ituarte 1989, Dreher-Mansur and Ituarte 1999). In the past these genera have been divided into three subfamilies: Euperinae, Pisidiinae, Sphaeriinae. The Euperinae are comprised of *Byssanodonta* and *Eupera* (Heard 1965b, Dreher-Mansur and Meier-Brook 2000) and diagnosed by (1) having a functional byssal gland in adults, (2) having well-separated branchial and anal siphons, and (3) lacking a brood-sac for developing embryos (Heard 1965b). They have restricted geographic distribution. *Byssanodonta,* a monotypic genus, is restricted to only a small portion of the upper Paraná River, Argentina (Dreher-Mansur and Ituarte 1999). *Eupera* occurs in Central and South America and Africa/Madagascar, although one species, *Eupera cubensis* (Prime, 1865), is found in the southern United States (Heard 1965b, Mackie and Huggins 1976, Dreher-Mansur and Ituarte 1999).

Among the remaining three genera, *Musculium* and *Sphaerium* were considered to comprise the subfamily Sphaeriinae, separated from the Pisidiinae, which included *Pisidium* (Baker 1927). The Sphaeriinae were diagnosed by (1) having partially fused siphons, (2) having multiple brood-sacs for developing embryos, and (3) lacking a functional byssal gland in adults. The Pisidiinae were diagnosed by (1) having an anteriorly elongated shell, (2) having a greatly reduced branchial siphon (completely absent in some taxa), (3) having only a single brood-sac per brood, and (4) lacking a functional byssal gland in adults (Baker 1927, Heard 1965b, Burch 1975). However, recent studies (Cooley and Ó Foighil 2000, Dreher-Mansur and Meier-Brook 2000) have shown that the Sphaeriinae and Pisidiinae are not natural groups, and Dreher-Mansur and Meier-Brook (2000) lumped them into the subfamily Sphaeriinae. *Musculium, Sphaerium,* and *Pisidium* are cosmopolitan genera with maximum diversities in the Holarctic Region and each genus contains numerous species (Burch 1975, Kuiper 1983).

Even though sphaeriid subgeneric classification and relationships are still poorly understood, a suite of character reductions apparently related to reduction in shell size occurs in the subgenera of *Pisidium.* The largest subgenus *Pisidium s. str.* has three mantle openings (a pedal slit, an anal siphon, and a reduced branchial opening) and both inner and outer demibranchs, each composed of two lamellae. With reduced shell size, outer demibranchs have only a single lamella in *Cyclocalyx* Dall, 1903, and the branchial mantle opening and outer demibranchs are completely lost in *Neopisidium* Odhner, 1921 (Odhner 1921, Heard 1966). Kuiper (1962) further divided *Neopisidium* into Gondwanan *Afropisidium* Kuiper, 1962 and Eurasian *Odhneripisidium* Kuiper, 1962, the former with a protruding ligament, the latter with an introverted ligament.

Sphaeriid reproduction and development

Sphaeriid clams have complex reproductive and developmental characteristics, some of which may represent adaptive specializations to freshwater environments. All sphaeriid clams studied to date are simultaneous hermaphrodites (Woods 1931, Okada 1935a, Heard 1965a, Ituarte 1997, Araujo and Ramos 1997). Sexual maturation occurs remarkably early in sphaeriid ontogeny (Burky 1983, Holopainen and Hanski 1986), and in some cases, gametogenesis is initiated prior to release from the parental clam (Heard 1977). Whereas the male portion takes up the greater part of the gonad in fully-grown individuals of *Musculium, Pisidium,* and *Sphaerium* (Okada 1935a, Heard 1977, Araujo and Ramos 1997), the ovarian portion is much larger than the testicular portion in *Byssanodonta* and *Eupera* (Ituarte 1997, Dreher-Mansur and Meier-Brook 2000). Histological (Okada 1935c, Araujo and Ramos 1997, 1999), experimental (Odhner 1921, 1929, Thomas 1959, Meier-Brook 1970), and allozyme (Hornbach *et al.* 1980b, McLeod *et al.* 1981) studies have indicated self-fertilization in the Sphaeriidae, and the hermaphroditic duct (Okada 1935a, Meier-Brook 1970), inner demibranchs (Araujo and Ramos 1999), and gonads (Araujo and Ramos 1997) have been suggested as sites of fertilization.

All sphaeriid clams brood their direct-developing young within the inner demibranchs until they are released as benthic juveniles (Gilmore 1917, Okada 1935b, Bonetto and Ezcurra 1964, Heard 1965a, 1965b, 1977, Mackie *et al.* 1974b, Ituarte 1997). Sphaeriid genera, however, show different degrees of complexity in the details of how brooding is achieved. The simplest form is found in euperine species. Ripe eggs in euperine taxa are large (200-400 μm in diameter) and have a large amount of yolk. It is generally believed that the euperine embryos are nourished mainly by yolk during development (Ituarte 1997, Dreher-Mansur and Ituarte 1999, Dreher-Mansur and Meier-Brook 2000). All embryos in a brood are synchronously spawned and developed as a single cohort. Developing embryos lie between the ctenidial (gill) lamellae without any link to the parental tissues (Heard 1965b, Mackie and Huggins 1976, Dreher-Mansur and Ituarte 1999, Dreher-Mansur and Meier-Brook 2000).

Species of *Pisidium* are also synchronous brooders. Yet, unlike *Byssanodonta* and *Eupera,* their embryos develop within a distinct brood-sac (marsupial-sac), which is formed by an outgrowth of the descending filaments of inner demibranchs (Heard 1965a, 1977, Mackie *et al.* 1974b). In contrast, species of *Sphaerium* and *Musculium* are sequential brooders, *i.e.,* multiple subsets of embryos in discrete ontogenetic stages are simultaneously present within the inner demibranchs. Each subset results from a distinct spawning event and is sheltered in a separate brood-sac (Heard 1977, Mackie 1979). Sphaeriine eggs are much smaller (about 100 μm in diameter) and have less yolk than those of euperine species (Raven 1958, Mackie 1978a, Beekey *et al.* 2000). Transfer of nutrition from the gill to the embryo during development has been indicated in some *Musculium* species based on detailed histological, cytochemical, and ultrastructural analyses (Okada 1935b, Hetzel 1994). In addition, a high brood mortality rate observed in many sphaeriine species (Avolizi 1976, Mackie *et al.* 1976, Meier-Brook 1977, Hornbach *et al.* 1980b, 1982, Mackie and Flippance 1983) may indicate that successfully developing embryos are nurtured by the less successful ones (Avolizi 1976).

Conflicting views of sphaeriid evolution

The systematic validity and relationships of sphaeriid genera are controversial although some conclusions have been traditionally held. Early taxonomic studies have suggested sister-group relationships between *Byssanodonta* and *Eupera* (Klappenbach 1960, Ituarte 1989) and between *Musculium* and *Sphaerium* (Burch 1975, Heard 1977, Hornbach *et al.* 1980a) based on morphological and reproductive/developmental characters. Indeed, many workers (Sterki 1909, Thiele 1934, Haas 1949, Ellis 1962, Herrington 1962, Bowden and Heppell 1968, Gale 1972, Clarke 1973, Dreher-Mansur and Ituarte 1999) have questioned whether or not they are sufficiently distinct to warrant separate generic status. A number of malacologists (Meier-Brook 1970, 1977, Korniushin 1998a, 1998b, 1998c, Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002) have recognized a reduction of shell and body size accompanied by a series of conspicuous anatomical character losses and/or simplifications in smaller-sized *Pisidium* taxa, and assumed that miniaturization represents the major evolutionary trend in the Sphaeriinae. However, these phylogenetic hypotheses were not tested until very recently.

Using morphological characters, Dreher-Mansur and Meier-Brook (2000) and Korniushin and Glaubrecht (2002) performed cladistic analyses of the Sphaeriidae, but only the later study tested monophyly of the sphaeriid taxa and robustness of the recovered clades. Both analyses found two well-supported clades, Euperinae (*Eupera*, *Byssanodonta*) and Sphaeriinae ((*Sphaerium* (*Musculium*, *Pisidium*)), and only *Pisidium* was recovered as monophyletic (Korniushin and Glaubrecht 2002), being a derived clade among sphaeriine genera (Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002). In both studies, a sister-group relationship between *Musculium* and *Pisidium* was supported based primarily on a suite of micro-scale kidney characteristics. This result, however, was very weakly supported (bootstrap value was less than 50% [Korniushin and Glaubrecht 2002]) and incongruent with the earlier taxonomic studies contesting the generic distinctiveness of *Musculium* from *Sphaerium* (Sterki 1909, Ellis 1962, Herrington 1962, Bowden and Heppell 1968, Gale 1972, Clarke 1973). Although Korniushin and Glaubrecht (2002, Fig. 1B) further suggested some changes, mainly the elevation of taxonomic ranks, to the classification of the Sphaeriidae, many of these changes lack topological and statistical support.

Cooley and Ó Foighil (2000) generated the first comprehensive sphaeriid gene tree using mitochondrial 16S rDNA sequences. The taxon sampling effort was expanded to incorporate nuclear (ITS1 RNA) and mitochondrial (16S RNA) genomes in Lee and Ó Foighil (2003). Both studies yielded a paraphyletic *Pisidium* in which the subgenus *Afropisidium* was sister to all the other sphaeriine taxa considered, either alone (16S data) or together with the *Odhneripisidium* (ITS1-containing data sets). Asynchronous brooders (*Sphaerium* and *Musculium*) consistently formed a derived monophyletic group within the Sphaeriinae and it was strongly supported in a combined analysis of 16S and ITS1 (Lee and Ó Foighil 2003). Basal *Pisidium* paraphyly and derived sequential brooder monophyly are also apparent in preliminary trees based on nuclear gene fragments: 28S ri-

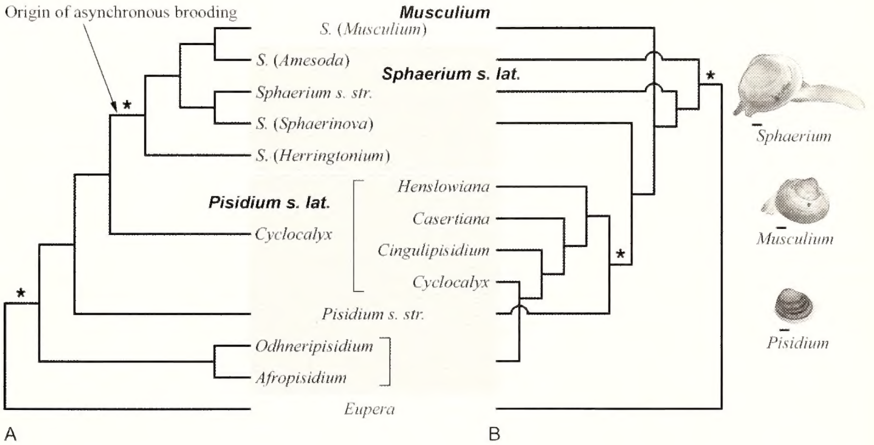

Figure 1. Two competing hypotheses of sphaeriid phylogeny. A, Hypothesis of Lee and Ó Foighil (2003) based on molecular (16S and ITS1) sequence data. B, Hypothesis of Korniushin and Glaubrecht (2002) based on morphological characters. While gene trees showed a paraphyletic *Pisidium* and a derived monophyletic clade of asynchronous brooders (*Sphaerium* and *Musculium* taxa), morphological analyses recovered a derived monophyletic *Pisidium* clade and a sister relationship among *Musculium* and *Pisidium* taxa. An "*" indicates a strong support (bootstrap value >90%) and scale bars represent 2 mm.

bosomal RNA (Park and Ó Foighil 2000) and 6-phosphogluconate dehydrogenase (Lee and Ó Foighil 2002). Sphaeriine classification was also revised based on molecular analyses, and five robust clades (*Afropisidium*, *Odhneripisidium*, *Pisidium*, *Cyclocalyx*, and *Sphaerium*) were suggested as generic groupings (Lee and Ó Foighil 2003, Fig. 1A).

There are two major topological incongruencies between molecular- and morphology-based sphaeriid phylogenies. While a derived monophyletic *Pisidium* clade was strongly supported and diagnosed by a number of morphological characters (Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002), independent as well as combined analyses of various gene sequence data consistently yielded paraphyletic *Pisidium* (Cooley and Ó Foighil 2000, Park and Ó Foighil 2000, Lee and Ó Foighil 2002, 2003). The other pointed element of incongruence concerns the phylogenetic relationships among the synchronous (*Eupera* and *Pisidium*) and asynchronous (*Sphaerium* and *Musculium*) brooding taxa. Molecular studies recovered a derived monophyletic clade of asynchronous brooders (*Eupera* (*Pisidium* (*Musculium*, *Sphaerium*))), but morphological analyses yielded a (*Eupera* (*Sphaerium* (*Musculium*, *Pisidium*))) topology (Fig. 1). The phylogenetic placement of the asynchronous brooders is important because it shapes our view of the primary evolutionary trends in the Sphaeriinae. According to morphological studies, the characters observed in the larger *Sphaerium* taxa are mostly plesiomorphic and derived from those are the reduced *Pisidium*, especially the subgenus *Neopisidium*, characters. Conversely, molecular data consistently place *Sphaerium* in a derived sphaeriine clade together with *Musculium*, revealing the evolutionary elaboration of brooding character complexity from synchronous to sequential brooding.

The robust topological congruence among independent molecular trees based on diverse gene fragments suggests that a careful reexamination of morphological data is warranted. Therefore, the morphology and life history of North American sphaeriid taxa was studied, widely accepted characters were newly coded, and a parsimony analysis was performed. The results showed that *Sphaerium* and *Musculium* taxa form a monophyletic group, congruent with previous gene trees but not with morphological studies. The *Pisidium* monophyly conflict between molecular and morphological trees, however, remains unsettled. At present, informative morphological characters appear insufficient to flesh out phylogenetic relationships among infra-generic sphaeriid taxa.

MATERIALS AND METHODS

Taxa examined

Of the five widely recognized sphaeriid genera, one to six representatives of all four North American genera (*Eupera*, *Musculium*, *Pisidium*, and *Sphaerium*) were chosen for analyses. Seventeen species selected were also representatives of all sphaeriid subgenera recognized in North America (Table 1). Herein I use the classification system of Burch (1975) and Dreher-Mansur and Meier-Brook (2000), instead of newly suggested ones (Korniushin and Glaubrecht 2002, Lee and Ó Foighil 2003), to test their competing hypotheses. The marine *Astarte sulcata* (Da Costa, 1778) and the freshwater *Corbicula* North American "*Form A*" (for form designation see Siripattrawan *et al.* 2000) and *Neocorbicula limosa* (Maton, 1809) were also included as outgroups in order to root the sphaeriid phylogeny. It has been widely held that the Astartidae have many plesiomorphic morphological characters, occupying a basal position within the order Veneroida (Taylor *et al.* 1973, Morton 1996). Indeed, the *Astarte* species was sister to all the other heterodont taxa studied, including sphaeriids, in a recent molecular phylogenetic analysis (Park and Ó Foighil 2000). The fresh/brackish water family Corbiculidae has long been placed in the superfamily Corbiculoidea together with Sphaeriidae (Newell 1965, Keen and Casey 1969, Taylor *et al.* 1973, Boss 1982, Morton 1996) although their sister-relationship was rejected recently (Dreher-Mansur and Meier-Brook 2000, Park and Ó Foighil 2000). In addition, the two corbiculid species chosen display a number of specialized reproductive and developmental characters similar to the ingroup taxa. Even though I recognize this apparent convergent evolution as a potential problem, convincing sister taxa for the Sphaeriidae have not yet been identified. Thus, an unrooted analysis using the ingroup taxa only was conducted in order to check if outgroup rooting generates any phylogenetic conflict.

Characters

Character states for each species were determined from the examination of shells and alcohol-preserved specimens deposited in the University of Michigan, Museum of Zoology and from the previous studies (see references in Table 1). Only the diagnostic characters accepted widely by sphaeriid systematists were coded. Micro-scale anatomical characters used in previous cladistic analyses (Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002) were not included when they were phylogenetically uninformative and/or controversial among studies. Inapplicable characters, such as branchial siphon characters in the species with no branchial siphon, were coded as dashes (-) and a missing character with a question mark (?). The character matrix is shown in Table 2.

Characters and character states (an asterisk below denotes characters that appeared in Korniushin and Glaubrecht [2002], their character states were determined independently in the present study).

Table 1. Catalog of the studied taxa, information on specimens examined (Mollusk Division Catalog Number, University of Michigan, Museum of Zoology), and references to anatomical and life-history characters. Specimens were sampled by [1]William H. Heard, [2]Cristián F. Ituarte, [3]Renée S. Mulcrone, [4]Mary Yong, and [5]the author.

Taxon	Collection locality	Catalog number	References
Family Astartidae d'Orbigny, 1844			
Genus *Astarte* J. Sowerby, 1816			
**A. sulcata* (Da Costa, 1778)	England	9700	Saleuddin 1964, 1965, Boss 1982
Family Corbiculidae Gray, 1847			
Genus *Corbicula* Megerle, 1811			
***C.* (North American "*Form A*")	[3]Michigan, USA	266693	Britton and Morton 1982, King *et al.* 1986, Ituarte 1994, Dreher-Mansur and Meier-Brook 2000
Genus *Neocorbicula* Fischer, 1887			
N. limosa (Maton, 1809)	[2]Argentina	265500	Ituarte 1994, Dreher-Mansur and Meier-Brook 2000
Family Sphaeriidae Deshayes, 1854 (1820)			
Subfamily Euperinae Heard, 1965			
Genus *Eupera* Bourguignat, 1854			
E. cubensis (Prime, 1865)	[4]Cuba	266709	Heard 1965b, Mackie and Huggins 1976, Dreher-Mansur and Meier-Brook 2000
Subfamily Sphaeriinae Baker, 1927			
Genus *Musculium* Link, 1807			
**M. lacustre* (Müller, 1774)	[1]Michigan, USA	266756	Heard 1977, Dreher-Mansur and Meier-Brook 2000
	[5]Michigan, USA	266755	
M. partumeium (Say, 1822)	[1]Ontario, Canada	266757	Heard 1977
	[5]Michigan, USA	266670	
M. securis (Prime, 1852)	[1]Michigan, USA	266758	Mackie and Qadri 1974, Mackie *et al.* 1974a, 1974b, Heard 1977
	[5]Michigan, USA	266710	
M. transversum (Say, 1829)	[1]Ohio, USA	266721	Heard 1977
	[3]Michigan, USA	266722	
Genus *Pisidium* Pfeiffer, 1821			
Subgenus *Cyclocalyx* Dall, 1903			
P. adamsi Stimpson, 1851	[1]Michigan, USA	266764	Heard 1966
	[5]Michigan, USA	266663	
P. casertanum (Poli, 1791)	[1]Michigan, USA	266725	Heard 1965a, 1966
	[5]Michigan, USA	266728	
P. compressum Prime, 1852	[1]Michigan, USA	266729	Heard 1965a, 1966
	[5]Michigan, USA	266714	
P. variabile Prime, 1852	[1]Michigan, USA	266740	Heard 1965a, 1966
	[5]Michigan, USA	266665	
Subgenus *Neopisidium* Odhner, 1921			
**P. conventus* Clessin, 1877	[1]Michigan, USA	266742	Heard 1963, 1965a, 1966
Subgenus *Pisidium s.str.*			
P. dubium (Say, 1816)	[1]Michigan, USA	266746	Heard 1965a, 1966
	[5]Michigan, USA	266715	
Genus *Sphaerium* Scopoli, 1777			
Subgenus *Herringtonium* Clarke, 1973			
**S. occidentale* (Prime, 1856)	[1]Michigan, USA	266753	Clarke 1973, Heard 1977
	[5]Michigan, USA	266752	
Subgenus *Sphaerium s.str.*			
**S. corneum* (Linnaeus, 1758)	[1]Ontario, Canada	266760	Jacobsen 1828, Heard 1977, Dreher-Mansur and Meier-Brook 2000
S. fabale (Prime, 1852)	[1]Michigan, USA	266748	Heard 1977
	[5]Michigan, USA	266747	
S. rhomboideum (Say, 1822)	[1]Ontario, Canada	266763	Heard 1977
	[5]Michigan, USA	266762	
S. simile (Say, 1816)	[1]Michigan, USA	266750	Drew 1896, Gilmore 1917, Heard 1977
	[5]Michigan, USA	266712	
S. striatinum (Lamarck, 1818)	[1]Michigan, USA	266751	Monk 1928, Heard 1977
	[5]Michigan, USA	266679	

*: Type species of the genus and subgenus.

**: For form designation see Siripattrawan *et al.* (2000).

References for shell characters: Baker (1928), Herrington (1962), Clarke (1973), Burch (1975), Mackie *et al.* (1980).

Table 2. Data matrix used in the phylogenetic analysis.

										1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0
Astarte sulcata	0	0	0	0	0	0	0	0	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	0
Corbicula North American "*Form A*"	0	0	1	1	0	0	0	1	0	0	1	0	1	0	0	1	0	1	1	0	1	1	1	1	0	0	-	0	0	0
Neocorbicula limosa	0	0	1	1	0	0	0	1	0	0	1	0	1	0	0	1	0	1	1	0	1	1	0	1	1	0	-	0	0	?
Eupera cubensis	0	1	2	0	0	1	0	1	1	0	1	1	0	0	0	1	1	1	0	1	1	1	0	1	0	0	-	0	0	0
Musculium	0	2	2	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
Pisidium (*Cyclocalyx*)	1	2	2	0	0	0	0	0	1	-	1	1	0	0	1	1	1	1	0	0	1	1	0	1	0	1	0	0	0	1
Pisidium (*Neopisidium*)	1	2	2	0	0	0	1	-	1	-	0	-	0	1	-	1	1	1	0	0	1	1	0	1	0	1	0	0	0	1
Pisidium (*Pisidium*)	1	2	2	0	0	0	0	0	1	-	1	1	0	0	0	1	1	1	0	0	1	1	0	1	0	1	0	0	0	1
Sphaerium (*Herringtonium*)	0	2	2	0	1	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
Sphaerium (*Sphaerium*)	0	2	2	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	1	1	1	0	1	1
Sphaerium (*Sphaerium*) *corneum*	0	2	2	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1

Shell characters

*1. Shell shape: 0 = equilateral to posteriorly elongated, 1 = anteriorly elongated.

*2. Number of cardinal teeth in left valve: 0 = three, 1 = one, 2 = two.

*3. Number of cardinal teeth in right valve: 0 = two, 1 = three, 2 = one.

*4. Morphology of lateral teeth: 0 = smooth, 1 = serrate.

5. Inside of the shell: 0 = not ridged, 1 = ridged.

6. Adventitious maculations on inner shell surface: 0 = absent, 1 = present.

Gross soft-anatomy characters

*7. Number of mantle openings: 0 = three (pedal slit, branchial, and anal openings), 1 = two (pedal slit and anal openings).

*8. Branchial (incurrent) opening: 0 = not produced into elongate siphon, 1 = produced into elongate siphon.

9. Morphology of branchial and/or anal (excurrent) siphons: 0 = papillated, 1 = smooth.

*10. Branchial and anal siphons when extended: 0 = well separated, 1 = fused in part.

*11. Pallial fusion ventral to the branchial siphon or aperture (pre-siphonal suture): 0 = absent, 1 = present.

*12. Morphology of pre-siphonal suture: 0 = short, 1 = elongated.

13. Morphology of mantle edge ventral to the branchial or anal opening: 0 = smooth, 1 = papillated.

*14. Ctenidia: 0 = with outer demibranchs, 1 = without outer demibranchs.

*15. Outer demibranchs: 0 = with two lamellae, 1 = with one lamella.

16. Demibranchs behind the foot: 0 = not united, 1= united to each other.

*17. Height of ascending lamellae of inner demibranchs: 0 = as high as descending lamellae, 1 = lower than descending lamellae.

18. Association of ascending lamellae of demibranchs with the mantle and visceral mass, 0 = fused only anteriorly and connected posteriorly by cilia, 1 = fused along entire length or almost so.

*19. Type of stomach (Purchon 1987): 0 = type IV, 1 = type V.

*20. Functional byssus in adult: 0 = absent, 1 = present.

Brooding and life history characters

21. Habitat: 0 = marine, 1 = freshwater.

22. Sexual expression: 0 = gonochoristic, 1 = hermaphroditic.

23. Type of development: 0 = develop directly as juveniles without larval stage, 1 = develop indirectly with larval stage.

24. Extent of parental brooding: 0 = absent, 1 = present.

*25. Type of brooding habit: 0 = synchronous brooding (all embryos in a brood result from a single spawning event and develop as a single embryonic cohort), 1 = sequential brooding (the products of several distinct spawning events co-exist in the ctenidial marsupia and are composed of developmentally discrete subsets of embryos).

*26. Extent of brood-sac (marsupial-sac): 0 = absent, 1 = present.

27. Morphology of the brood sac: 0 = thick-walled and partitioned into smaller chambers, 1 = thin-walled without further partitioning (Heard 1977).

28. Fully developed juveniles retained within the

ctenidial marsupia (extra-marsupial larvae *sensu* Heard 1977): 0 = lying free within the marsupia, 1 = attached to the remnants of the brood sacs or to the descending lamellae of the inner demibranchs (Mackie *et al.* 1974a, Heard 1977).

29. Precocious maturation (production of gametes by brooded juveniles before being released from the ctenidia): 0 = absent, 1 = present (Heard 1977).

*30. Eggs: 0 = large with lots of yolk, 1 = small without sufficient yolk to nourish the embryo until maternal deposition of offspring.

Phylogenetic Analysis

The data were analyzed using PAUP* 4.0b8 (Swofford 2002) under the maximum parsimony optimality criterion. Analyses were performed as branch-and-bound searches using equal character weighting. All characters were treated as unordered and dashes were treated as missing data rather than as a new state. *Astarte sulcata*, *Corbicula* North American "*Form A*," and *Neocorbicula limosa* were designated as outgroups, and sphaeriid taxa were forced to be monophyletic to root the phylogeny. An unrooted analysis of ingroup taxa was also conducted and the results were compared with outgroup-rooted topology. Character transformation series were determined on one of the equally parsimonious trees using MacClade 3.07 (Maddison and Maddison 1997) and PAUP*. Branch support levels were calculated with bootstrapping (1000 replications, heuristic searches, 10 random additions each) using PAUP* and with Bremer Decay-Index values (Bremer 1994) using TreeRot (Sorenson 1999), which generates a constraint file for PAUP*.

RESULTS

Thirty characters were coded, including those from the shell (1-6), gross soft-anatomy (7-20), and brooding/life history (21-30). Within each subgenus, all representative species had the same character set except for *Sphaerium* (*Sphaerium*) *corneum* (Linnaeus, 1758), which had a different character state of fully developed juveniles retained within the ctenidial marsupia (Character 28) from the other *Sphaerium s. str.* taxa. To facilitate a thorough analysis, one character set from each subgenus was included in the datamatrix unless varied taxa existed (Table 1). Of 30 total, 18 characters were found to be parsimony-informative and only 7 were so in ingroup-only analysis.

Twelve equally most-parsimonious trees of 37 steps (CI = 0.865, RI = 0.844) were obtained from the analysis of the data matrix including outgroup taxa, and a strict consensus was recovered (Fig. 2). Sphaeriid genera formed a robustly supported monophyletic group and *Eupera cubensis* was sister to the monophyletic Sphaeriinae. *Musculium* was grouped with *Sphaerium* taxa, not with *Pisidium*, forming an unresolved polytomy, this result was fairly well supported by bootstrap value 85. Three North American *Pisidium* subgenera were recovered as monophyletic without further resolution and sister to the *Sphaerium s. lat.*/*Musculium* clade. The unrooted analysis of ingroup taxa alone resulted in the same topology (12 trees, L = 19, CI = 0.947, RI = 0.923, Fig. 3), but provided a much higher bootstrap value for the *Sphaerium s. lat.*/*Musculium* clade and the *Pisidium* clade. Character transformations were depicted on one of the most-parsimonious cladograms obtained from the outgroup-rooted analysis (Fig. 4).

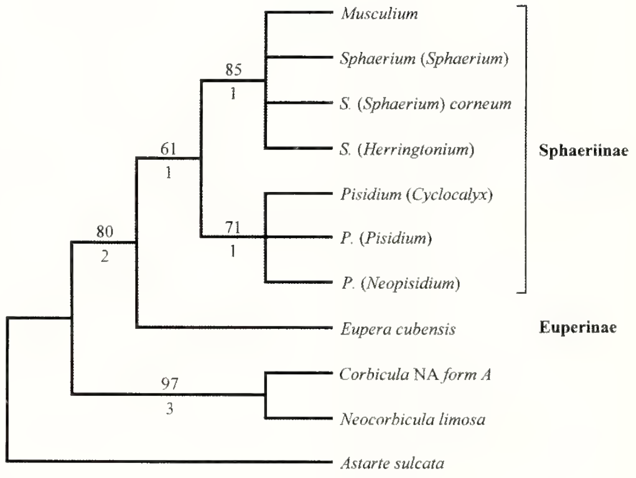

Figure 2. Strict consensus of 12 equally parsimonious trees (L = 37, CI = 0.865, RI = 0.844) obtained from the analysis including outgroup taxa. Numbers above and below internodes are bootstrap and Bremer values, respectively.

DISCUSSION

Previous morphological trees conflict with gene trees on two major elements of sphaeriid topology, *i.e.*, monophyly of *Pisidium* and of asynchronous brooders (*Sphaerium* and *Musculium*). The present study recovered a monophyletic *Pisidium* as in the other morphological analyses, but asynchronous brooders were found to be monophyletic as in the previous molecular studies (Figs. 2-3).

Lee and Ó Foighil (2003), the most comprehensive molecular study to date utilizing mitochondrial 16S and nuclear ITS1 sequences data, found four strongly supported clades within *Pisidium s. lat.*, and these terminal clades formed a paraphyletic assemblage (Fig. 1A). Paraphyletic *Pisidium* was also apparent in all the other gene trees generated by diverse nuclear and mitochondrial gene fragments: Mitochondrial 16S ribosomal RNA (Cooley and Ó Foighil, 2000), nuclear

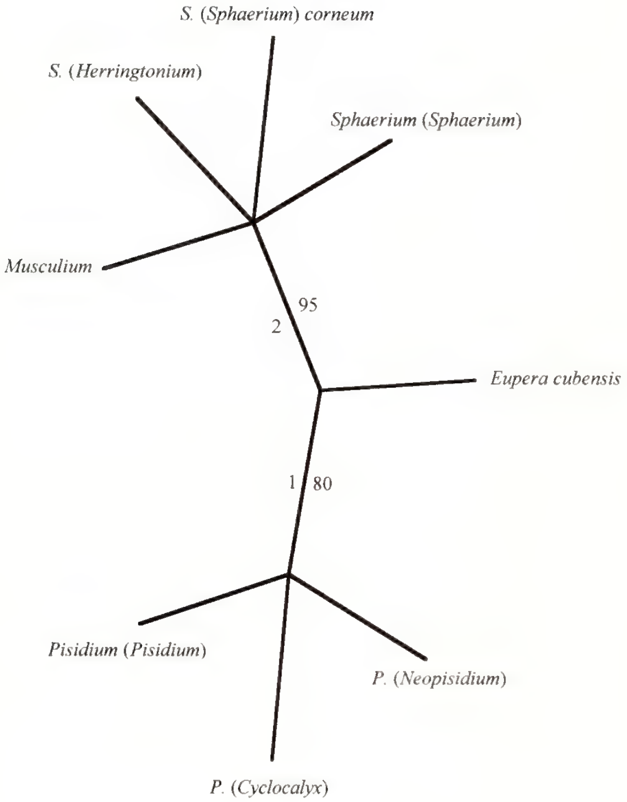
S. (Sphaerium) corneum
S. (Herringtonium)
Sphaerium (Sphaerium)
Musculium
95
2
Eupera cubensis
1
80
Pisidium (Pisidium)
P. (Neopisidium)
P. (Cyclocalyx)

Figure 3. Unrooted strict consensus of 12 equally parsimonious trees obtained from the analysis of ingroup taxa only (L = 19, CI = 0.947, RI = 0.923). Numbers right and left of internodes are bootstrap and Bremer values, respectively.

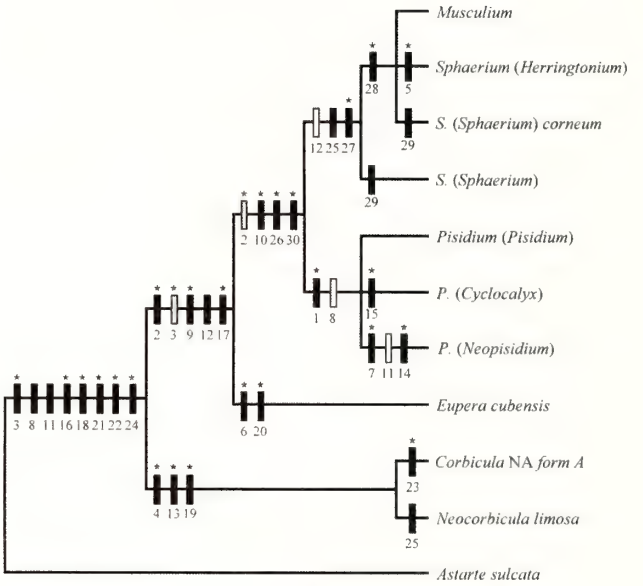
Musculium
Sphaerium (Herringtonium)
S. (Sphaerium) corneum
S. (Sphaerium)
Pisidium (Pisidium)
P. (Cyclocalyx)
P. (Neopisidium)
Eupera cubensis
Corbicula NA form A
Neocorbicula limosa
Astarte sulcata
28
5
12 25 27
29
29
2 10 26 30
1 8
15
7 11 14
2 3 9 12 17
6 20
3 8 11 16 18 21 22 24
23
4 13 19
25
*

Figure 4. One of 12 equally most-parsimonious trees rooted by outgroup showing character transformations. The numbers below the bars denote the character number changing along that internode. Character numbers refer to those listed in materials and methods. Black bars indicate character changes from 0 to 1, gray bars from 1 to 2, and white bars from 1 to 0. An "*" indicates an unambiguous character transformation.

28S ribosomal RNA (Park and Ó Foighil 2000), and nuclear single copy 6-phosphogluconate dehydrogenase (Lee and Ó Foighil 2002). Among the *Pisidium* terminal clades, *Afropisidium* was consistently positioned basally within the Sphaeriinae (Fig. 1A, Cooley and Ó Foighil 2000, Park and Ó Foighil 2000, Lee and Ó Foighil 2003). On the other hand, Korniushin and Glaubrecht's (2002) morphological analysis recovered a derived monophyletic *Pisidium s. lat.* clade (Fig. 1B). This was strongly supported by a bootstrap value of 98 and diagnosed by 8 shared derived characters, of which 4 were unambiguous. The present study confirmed *Pisidium* monophyly (Fig. 2). Six *Pisidium* species belonging to 3 North American subgenera formed a moderately supported (bootstrap value = 71) monophyletic group. This clade was diagnosed by at least two synapomorphies, anteriorly elongated shell shape (character 1) and reduced branchial siphon (character 8) (Fig. 4).

Given the strong support and/or consistency in both data sets, the disparity on *Pisidium* monophyly may originate from the actual differences between molecular and morphological phylogenies. However, there are some points that need to be addressed before such a conclusion can be made. First of all, this conflict may be attributable to undetected convergent evolution in sphaeriid morphology, although molecular sequence data are not free from homoplasy (Hillis and Wiens 2000). Most morphological synapomorphies for the *Pisidium* clade are losses and/or simplifications of anatomical characters such as outer demibranchs and siphons. If some of these character losses were linked to body size reduction and if miniaturization happened independently in each *Pisidium s. lat.* lineage, convergent apomorphies might be accumulated in these already well-separated paraphyletic lineages, preserving misleading phylogenetic information. In addition, sphaeriine lineages are apparently very old—*Afropisidium* has a Gondwanan distribution (Kuiper 1983). Thus, previous and the present morphological studies that have analyzed living taxa alone may have been misled. Secondly, taxon sampling was not complete in both data sets. Molecular studies lack any *Neopisidium* taxa and have a mere two species each of *Afropisidium* and *Odhneripisidium*. The number of *Neopisidium* taxa included was limited in Korniushin and Glaubrecht (2002) and the present morphological study was restricted to North American taxa, lacking any representative species of

Afropisidium and *Odhneripisidium.* An extensive taxon sampling effort will ultimately improve phylogenetic accuracy (Hillis 1998, Pollock *et al.* 2002, Zwickl and Hillis 2002).

A sister-relationship among asynchronous brooders (*Sphaerium s. lat.* and *Musculium* taxa) suggested by molecular studies was confirmed in the present morphological analysis (Figs. 2 and 3). According to the outgroup-rooted analysis, the *Sphaerium/Musculium* clade was supported by bootstrap value 85 and diagnosed by at least one unambiguous and two ambiguous synapomorphies: Short presiphonal suture (character 12), sequential brooding (character 25), and non-partitioned brood sac (character 27) (Fig. 4). When outgroups were excluded from the analysis, all these characters, together with character 10 fused siphon, unambiguously diagnosed the clade and the bootstrap value reached 95.

This result is not surprising because morphology-based sphaeriine phylogenies (*Sphaerium* (*Musculium*, *Pisidium*)) recovered previously (Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002) were far from robust. Dreher-Mansur and Meier-Brook (2000) suggested a sister-relationship for *Musculium* and *Pisidium* based on one shell and three kidney characters. However, three of these inferred synapomorphies appear to be controversial: 39, lack of the complex crossed-lamellar structure in the shell (see Mackie 1978b), 40, very long kidney funnel (see Korniushin and Glaubrecht 2002 character 47), and 42, slit-like opening of the excretory sac (see Korniushin and Glaubrecht 2002 character 58). They considered their character 49, reduced number of brood sacs, as an autapomorphy of *Pisidium*, but since only sphaeriine taxa have brood sacs, having multiple brood sacs would have been a synapomorphy supporting *Sphaerium/Musculium* clade as it is in the present study (character 27, Fig. 4). Dreher-Mansur and Meier-Brook (2000) also overlooked phylogenetically informative brooding characteristics, which have long been recognized as diagnostic characters for generic-level classification, *i.e.*, sequential/synchronous brooding patterns, morphology of brood sacs, and the position of developing embryos. In Korniushin and Glaubrecht (2002), only one character, narrow kidney funnel (character 48), ambiguously supported their *Musculium/Pisidium* clade, the other supporting character, one lamella outer demibranch in the incubated young (character 69), is actually an apomorphy for *Musculium* because this was inapplicable to *Pisidium* taxa. The supporting bootstrap value was less than 50% and the alternative topology (*Pisidium* (*Musculium*, *Sphaerium*)) was a mere one step longer (Korniushin and Glaubrecht 2002).

Methodological distinctions between the present and previous morphological studies are likely to underlie the differential topological results. While the present study restricted the character set to major anatomical and developmental features that have been considered fundamental by previous workers, Dreher-Mansur and Meier-Brook (2000) and Korniushin and Glaubrecht (2002) included a large number of fine-scale anatomical features, especially numerous, potentially non-independent details of kidney substructure, in their data sets. Although micro-scale anatomical structures have recently been utilized to address taxonomic problems of the Sphaeriidae mainly by malacologists of the Russian school (Korniushin 1991, 1994, 1995, 1998a, 1998b, 1998c, 1999, Piechocki and Korniushin 1994), the plasticity of delicate anatomical differences (Korniushin 1998a) makes the application of these characters to cladistic analyses very difficult at this stage. Because of the potential for significant convergent evolution in brooding character states in corbiculid outgroup taxa (Dreher-Mansur and Meier-Brook 2000, Park and Ó Foighil 2000), the present study analyzed ingroup characters without rooting in addition to outgroup rooted analysis. Dreher-Mansur and Meier-Brook (2000) and Korniushin and Glaubrecht (2002), however, included each one of *Corbicula* and *Neocorbicula* lineages as an outgroup and did not test for outgroup rooting problems. It is noteworthy that the same sphaeriid topology (Euperinae (*Pisidium* (*Sphaerium*, *Musculium*))) as the present study was recovered when the author analyzed Korniushin and Glaubrecht's (2002) character matrix without corbiculids.

A number of sphaeriid systematists (Meier-Brook 1970, 1977, Korniushin 1998a, 1998b, 1998c, Dreher-Mansur and Meier-Brook 2000, Korniushin and Glaubrecht 2002) have proposed that miniaturization represents the predominant evolutionary trend in the Sphaeriinae and that the conspicuous character reductions observed in smaller-sized *Pisidium* taxa are derived from larger *Sphaerium* as a consequence of shell and body size reduction. However, it is now clear that this evolutionary trend is largely restricted to *Pisidium* taxa, especially *Neopisidium.* The present analysis, instead, supports the evolutionary development in brooding character complexity from synchronous to sequential brooding (Korniushin 1991, Cooley and Ó Foighil 2000). Synchronous brooding observed in *Eupera* and *Pisidium* is plesiomorphic, rejecting the hypothesis that having a single brood sac per demibranch is derived from a multiple brood sac condition found in *Sphaerium* and *Musculium* due to the ctenidial size-reduction (Korniushin 1998b, Dreher-Mansur and Meier-Brook 2000).

Previous molecular and morphology-based phylogenies were concordant in that three cosmopolitan genera, *Pisidium*, *Musculium*, and *Sphaerium*, form a monophyletic group, the Sphaeriinae, with respect to *Eupera* species. The present study also supports this conclusion. *Eupera* was segregated from the other sphaeriid clades early at the basal node, and the Sphaeriinae were diagnosed by four unambiguous synapomorphies (Fig. 4): Two cardinal teeth in the left valve (character 2), fused siphons (character 10, this is a synapomorphy supporting the *Sphaerium*/*Musculium* clade when ingroup taxa only were analyzed), the incubation of developing embryos within brood sacs (character 26), and non-yolky eggs (character 30).

The sister-group relationship among asynchronously brooding *Sphaerium* and *Musculium* taxa, supported by molecular studies (Cooley and Ó Foighil 2000, Park and Ó Foighil 2000, Lee and Ó Foighil 2002, 2003) and traditionally held by many sphaeriid systematists (Ellis 1962, Herrington 1962, Bowden and Heppell 1968, Clarke 1973), was confirmed by the present analysis of the morphological characters considered fundamental by previous workers. However, the *Pisidium* monophyly conflict between molecular and morphological trees remains unsettled. In addition, this study was not able to provide further infra-generic level relationships: Both *Sphaerium s. lat.*/*Musculium* and *Pisidium* clades were polytomous. This may be due to the lack of informative morphological characters. Only seven of a total 30 characters were parsimony-informative in ingroup comparison and no synapomorphy supporting *Musculium* and *Pisidium s. str.* was found. A sufficient number of characters, of whatever type, as well as taxa are crucial in estimating phylogeny (Hillis and Wiens 2000). Adding fossil characters to morphological analyses and incorporating more slowly evolving DNA sequences in the present molecular data set may provide sound basal sphaeriid relationships by redeeming ancestral states. Combined analysis of molecular and morphological data collected from global taxonomic sampling appears to be another challenging step toward a comprehensive phylogeny of the Sphaeriidae. To do this extensively, identification of convincing marine outgroups as well as a balanced taxon sampling among molecular and morphological data sets and among euperine and sphaeriine lineages are warranted.

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Use of a natural river water flow-through culture system for rearing juvenile freshwater mussels (Bivalvia: Unionidae) and evaluation of the effects of substrate size, temperature, and stocking density

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Abstract: The feasibility of rearing juvenile freshwater mussels using a culture system supplied with natural river water was investigated. Newly transformed juvenile rainbow mussels (*Villosa iris*) were reared for approximately 90 days in a flow-through culture system designed to simulate a stream channel. A total of 755 juveniles were reared to at least 90 days old over the course of 3 years. Juveniles were placed in containers partially filled with sieved river substrate, providing both a feeding medium and some protection from physical disturbance. Substrate size and depth were evaluated for influences on growth and survival of juveniles. Juvenile mussels in smaller substrate (<120 μm) grew slightly larger than those in larger substrate (between 120 and 600 μm) during one trial (reaching 2.22 mm *vs.* 1.97 mm in length, respectively [$p < 0.10$] from a starting size of approximately 0.30 mm), with no difference in survival. Substrate depth, 5 mm or 20 mm, had no effect on either survival or growth. In all experiments, most juveniles were found in the loose, flocculent layer of sediment brought in by the river water. The season when rearing of juveniles was begun had a significant effect on growth and survival of the mussels. Growth and survival were best when rearing was initiated in June and declined as rearing began later in the summer. Differences in water temperature of the culture system explained much of this variation. Separate laboratory experiments suggested that juvenile mussels stopped growing at temperatures below 15°C. When growth data were normalized for degree-days above 15°C, most of the variability in growth was explained ($R^2 = 0.88$, $p < 0.001$). The use of an *in situ* culture system with river water was shown to be feasible, but seasonal variables must be accommodated.

Key words: Unionidae, freshwater mussels, *Villosa iris*, flow-through aquaculture, juvenile mussels

The culture of juvenile freshwater mussels began in the early 20th century to augment populations in the Mississippi River basin that were being harvested for shells to make buttons for clothing. Howard (1916) was successful in culturing juvenile yellow lampmussels (*Lampsilis radiata luteola* Lamarck, 1819) to adulthood and obtained some second generation juveniles. However, other culture trials were not successful (Corwin 1920) and the specific techniques used by the early studies were not clearly reported. Mussel culture efforts were suspended thereafter until efforts to propagate endangered mussels developed in the 1980's.

To assist in the recovery of the 70 federally listed species in the U.S., the National Native Mussel Conservation Committee (1998) identified the propagation of juvenile mussels as an important component of the recovery plan for unionid species. Efforts to rear juvenile mussels to a size large enough to avoid many of the perils of early life in the wild were renewed in the last decade. Gatenby *et al.* (1997) investigated the survival and growth of juvenile rainbow mussels (*Villosa iris* [I. Lea, 1829]) reared in laboratory upweller dishes, showing that juvenile mussels could be cultured with artificial food and water sources. The effect of algal diet composition and sediment also was tested, confirming that juvenile mussels had specific nutritional needs that were not fully met with just a single food source (Gatenby *et al.* 1996). Yeager *et al.* (1994) showed that juvenile rainbow mussels need a suitable substrate in which to burrow and feed. Successful culture trials with young mussels also have been conducted in Europe using the pearl mussel (*Margaritifera margaritifera* [Linnaeus, 1758]) (Buddensiek 1995). All of these studies have focused on the goal of developing techniques necessary to make the culture of juvenile mussels a practical component of conservation.

Comparing results of culture trials using natural river water to those using laboratory water suggests that culture systems with natural river water have a higher potential for success. Results from early studies of the propagation of juvenile mussels suggest that the best methods are those using natural waters (Howard 1923). Further support for the efficacy of rearing juveniles in stream water is provided by Buddensiek (1995); juveniles held in river water had high survival rates (up to 20% after 12 months) and good growth. The long-term survival rates achieved with culture systems supplied by natural river water exceed those of laboratory-based systems (Howard 1923, Hudson and Isom 1984, Buddensiek 1995, Gatenby *et al.* 1997). These studies clearly indicate that a culture system supplied with natural river water should have a high probability of success.

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The objectives of this study were to evaluate the feasibility of using a flow-through culture system with natural river water for rearing juvenile mussels and to determine the effects of substrate size, substrate depth, and time of year on survival and growth of juvenile mussels.

MATERIALS AND METHODS

Glochidia of rainbow mussels (*Villosa iris*) were transformed to juveniles on rock bass (*Ambloplites rupestris* Rafinesque, 1817) in the laboratory. Gravid female mussels were collected from Copper Creek near Nickelsville in Scott County, Virginia, during May 1993 and on 27 May 1994, and 18 and 23 May 1995. Glochidia were flushed from the marsupia using a 5 ml syringe with a 3.8 cm, 21 gauge needle filled with conditioned municipal water (aerated for at least 24 hr with airstone). The glochidia were collected in a petri dish and placed in a 19 l bucket with 15 cm of conditioned municipal water. Three to five rock bass were placed in the bucket for 45 min, and an airstone was placed in the bucket to suspend the glochidia. After the 30-45 min exposure time, the fish were placed in separate 39 l static aquaria until the glochidia transformed and excysted, usually 13-20 days at 23°C. Transformed juveniles were collected by light siphoning pressure and filtration through a 120 μm sieve within 2 days after excystment. Newly transformed juveniles were held in 7.5 × 7.5 × 3.3 cm polypropylene dishes. The dishes were filled with a 1:1 mixture of conditioned municipal water and well water with a 3-5 mm layer of fine silt (particle size <120 μm) from the Clinch River immediately upstream of the American Electric Power Clinch River Steam Plant (CRSP) at Carbo, Russell County, Virginia, at river mile 266.1. Within 7 days, the juveniles were placed in the experimental culture systems.

The first experiment of this study was conducted in a flow-through culture system with ambient river water on the property of the CRSP. The water for the culture system was taken from the Clinch River immediately upstream of the power plant (pH 7.7-8.4, hardness 124-174 mg $CaCO_3$/l). Mussel populations in this upstream reach of the river appeared to be healthy and reproducing, with young adults present, such that water quality was deemed suitable for rearing juvenile mussels.

Water from the river was introduced into a U-shaped channel, which served as the flow delivery system for the oval troughs in which the juvenile mussels were reared (Fig. 1A). The channel also served as a settling chamber to remove much of the sediment introduced into the system. This channel had eleven holes drilled through each side at the same height. Each of the 22 holes was fitted with a short tube to guide the flow of water out of this channel and into the oval troughs where the mussels were held. Therefore, the channel served as both a flow regulator and a sediment trap.

The flow from the U-channel was directed into oval troughs, capable of holding approximately 75 l of water (Fig. 1B). Each of these troughs was equipped with a raised center that extended above the water level and a standpipe drain to regulate water level. The flow of water into these troughs was directed along the long axis of the oval to promote continuous current in a circular fashion. In addition, each of the oval troughs was fitted with a motorized paddle wheel to maintain unidirectional flow. Water velocity ranged from <1-20 cm/sec with a mean of 4.7 (sd = 4.3). This flow simulated the continual current experienced by mussels in a natural riverine setting.

Within these troughs, 50 newly transformed juvenile mussels were placed in each 75 × 50 × 50 mm rectangular glass container (during 1993) or 75 × 75 × 33 mm plastic container (during 1994 and 1995). Each of these containers was initially set up with one of two substrate types based on particle sizes. Sieves were used to obtain substrates with two particle size distributions, <120 μm and between 120 and 600 μm. The two substrates were tested for suitability in rearing juvenile rainbow mussels. In addition, two depths of each substrate were tested, 5 mm and 20 mm. Raw substrate was collected from the Clinch River immediately upstream of the CRSP for the 1993 and 1995 trials and from the Clinch River at Nash Ford for the 1994 trial. The use of river substrate provided a natural flora and meiofauna for the culture system. Since the native flora and meiofauna was a desired component of the culture substrate, heat or chemical treatments that would kill any potential predators and competitors were not used. Once the juveniles were settled in the substrate, the maintenance of a continuous current ensured that the mussels were exposed to a constant supply of suspended food and fresh water.

Juvenile growth and survival were assessed at the end of the growing season during 1993 (June-October) and 1994 (June-September for batch 1, September-November for batch 2), and at 30, 74, and 94 days during the 1995 trial (August-October). The sampled dishes were removed from the culture troughs and brought back to the Virginia Tech Aquaculture Center to be sampled. The substrate from the dishes was washed through a sieve, leaving the juvenile mussels and empty shells. During the 1995 trial, the substrate was sieved and searched in two fractions, the loose flocculent top layer and the more consolidated substrate underneath. The collected juveniles were counted, and the lengths and

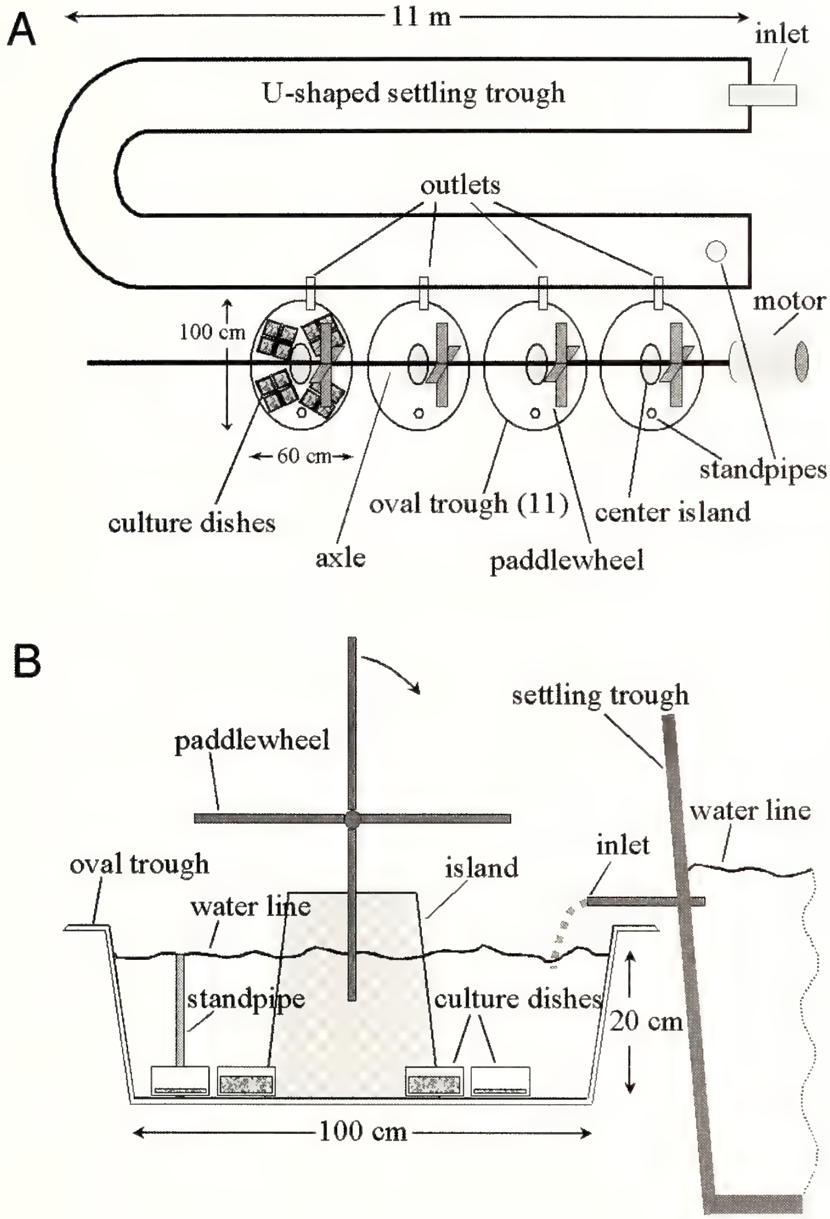

Figure 1. A, Diagram of the natural river water culture system; juvenile rainbow mussels were placed in 56 cm^2 dishes in the oval troughs, 16 dishes per trough; the electric motor turned paddlewheels to establish a unidirectional flow; river water continuously flowed through the troughs from the settling trough and out standpipes. B, Cross-sectional view of natural river water culture system; a constant flow of river water was delivered from the settling trough and water levels in the oval culture troughs were maintained by the standpipes; culture dishes were not placed under the paddlewheel, due to turbulence.

widths of a subset of 10-15 living juveniles, or of the entire set if less than 10, were measured using an ocular micrometer in a Zeiss binocular dissecting microscope. The remaining empty shells from mussels that died were counted to determine whether juveniles had died or escaped and examined to estimate the time of mortality based on size or number of growth rings. Comparison of the effect of substrate size, substrate depth, and year on survival and growth was conducted using the t-test procedure with $\alpha = 0.05$. The ANOVA procedure with $\alpha = 0.05$ was used to assess the effect of time on juvenile survival during the 1995 trial.

A second experiment to investigate the effect of temperature on the growth and survival of juvenile mussels was conducted in environmental chambers in the laboratory using 570 l Living Streams fitted with chiller units (Frigid Units, Inc., Toledo, Ohio) that provided a unidirectional flow, aerated water, and controlled temperature (Fig. 2). Heaters were placed in one stream to maintain a constant temperature of 25°C. The bottom of each stream was lined with washed pea-sized gravel to provide a substratum for natural algal and bacterial growth to occur. The streams were filled with Clinch River water approximately 2 weeks before the initiation of the experiment to allow colonization of natural flora. The three streams were maintained at 12°C, 18°C, and 25°C, to simulate late fall/early spring, mid-spring, and summer river temperatures, respectively. Light regime for all three streams was 12 hr light and 12 hr dark with the exception that the 25°C stream was covered with opaque foam approximately half of the days to reduce the growth of filamentous algae in the culture dishes. Juveniles were placed in these streams in 75 × 75 × 33 mm plastic containers filled to a depth of 10 mm with fine sediment (<120μm). The 18 containers per treatment were placed in the Living Streams with 50 juvenile mussels per container. Subsamples were taken at 30 and 60 days thereafter to determine growth and survival of juveniles at each temperature. Water that evaporated was replaced with distilled deionized water, and the experiment was terminated after 60 days. Statistical comparisons of the effects of temperature on growth and survival were performed using ANOVA with $\alpha = 0.05$.

The effect of stocking density on the survival and growth of juvenile rainbow mussels was tested using an ad-

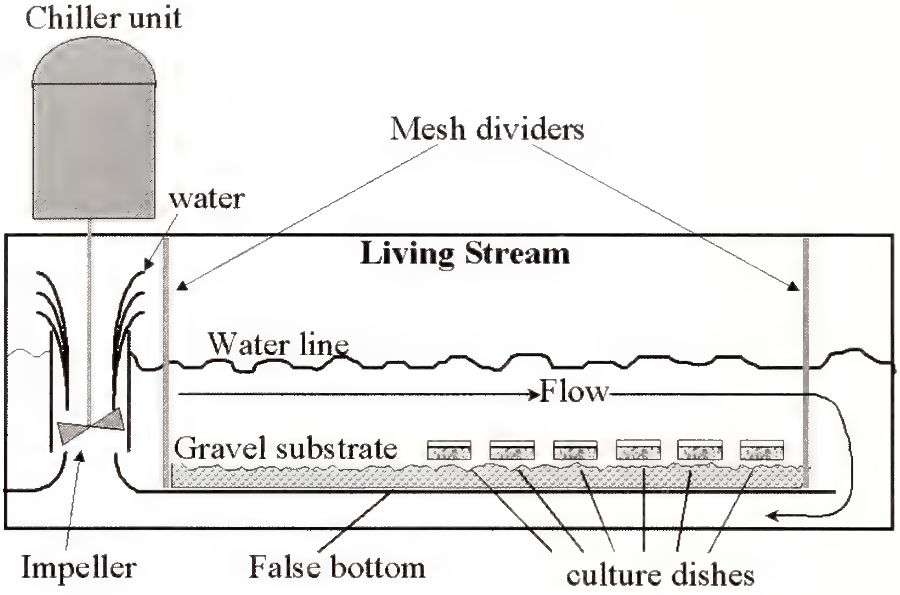
Chiller unit
Mesh dividers
water
Living Stream
Water line
Flow
Gravel substrate
Impeller
False bottom
culture dishes

Figure 2. Diagram of the controlled temperature experimental apparatus. Each of three systems was operated at a different temperature, 12°C, 18°C, or 25°C. Juvenile rainbow mussels were held in 18 dishes per system at a density of 50 mussels per 56 cm^2. Dishes were filled with fine sediment (<120µm) to a depth of 10 mm prior to introduction of juveniles.

ditional laboratory recirculating system constructed to simulate the Living Stream, generating a unidirectional current by pumping water from underneath a false bottom (Fig. 3). In this system, a false bottom was fitted into a 90 l tank with approximately 2 cm between the downstream end of the false bottom and the end of the tank. A small, external water pump was placed on the wall of the tank to circulate the water. Recirculated water was dispersed at the surface of the tank by means of a distribution header, consisting of a tank-wide, 12.5 mm diameter PVC pipe with drilled holes. Acid-washed rectangular plastic containers were filled with fine sediment (<120 µm) to a depth of 10-15 mm. Three sizes of containers were used, 75 × 75 mm, 130 × 75 mm, and 130 × 130 mm. Photoperiod was fixed at 14 hr light and 10 hr dark. At the start of the experiment, 6 replicates of each size dish, with 100 juveniles in each, were placed in the culture tank. The tank was filled with an equal mix of conditioned municipal water and well water to obtain a total hardness of 200 mg/l of $CaCO_3$. The juveniles fed on the natural flora and detritus that developed in the sediment because no regular supplements of algae were provided to the system. Water temperatures ranged from 22°C to 27°C and were allowed to follow ambient room temperature because it remained suitable for juvenile mussel culture. The experiment was allowed

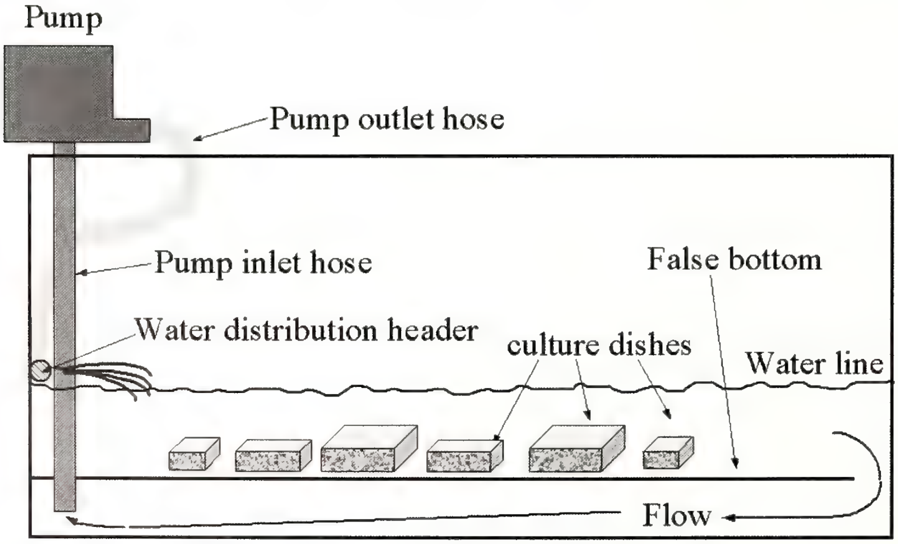
Pump
Pump outlet hose
Pump inlet hose
False bottom
Water distribution header
culture dishes
Water line
Flow

Figure 3. Diagram of the culture density experimental apparatus. One hundred juvenile rainbow mussels were placed in 6 replicate dishes of 3 sizes to achieve densities of 1.8, 1.0, and 0.6 juveniles per cm^2.

to run for 90 days; then containers were removed from the system, juveniles were sieved from the sediment, and growth and survival were determined. Statistical comparisons of the effects of stocking density on growth and survival were performed using ANOVA with $\alpha = 0.05$.

Comparisons among growth data from our study and others were based on cumulative degree-days. Fish hatchery managers have used this concept for predicting growth over the range of water temperatures in their facilities (Piper *et al.* 1982). The Monthly Temperature Units used in hatchery management are based on the average monthly water temperature above 0°C. For our study, comparisons were based on the cumulative degree-days above 15°C, a temperature below which juvenile mussels apparently do not grow appreciably. Regression analysis was used to determine the expected relationship between cumulative degree-days above 15°C and length. Outliers were excluded from the calculation of the regression equation based on residuals analysis ($p < 0.05$).

All statistical tests were performed using the statistical analysis software Statistica® (StatSoft 1998). Statistical significance levels were set at $\alpha = 0.05$ unless otherwise noted.

RESULTS

Growth and survival in natural river water flow-through culture system

During the 1993 trial, mean survival rate for juveniles was 24.3% and 21.8% to 115 days in the fine and coarse substrate, respectively (Table 1, Fig. 4). The initial sizes of juveniles were not measured, but all individuals were randomly taken from the same batch of newly transformed juveniles. Mean lengths of juveniles were marginally different between the fine and coarse substrates ($p < 0.10$), with juveniles reaching 2.22 mm (sd = 0.24) and 1.97 mm (sd = 0.35), respectively (Fig. 5). The variation in survival was high for the two treatments, 3-37% for the fine substrate and 1-50% for the coarse substrate. However, the fine substrate treatment exhibited more consistent survival rates. Depth of substrate had no effect on survival or growth. Asian clams were found in the dishes at the end of the experiment, with a mean of 8 clams per dish in the large substrate and 2 clams per dish in the fine substrate.

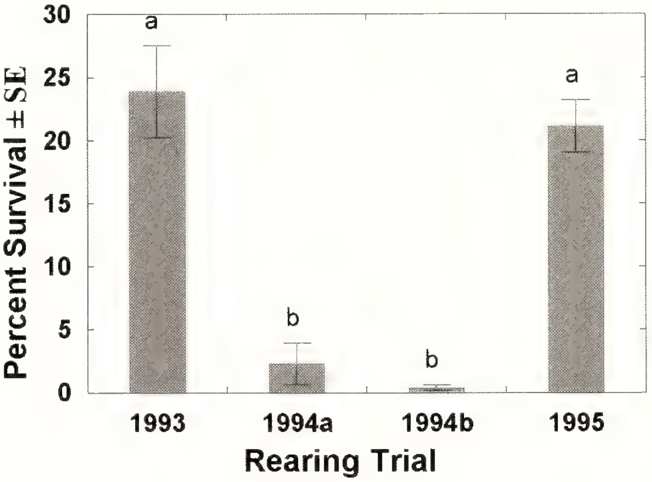

Figure 4. Mean percent survival per container of juvenile rainbow mussels (*Villosa iris*) for rearing trials in oval troughs. Error bars are ±1 SE and those with different letters are statistically different at the $\alpha = 0.05$ level.

Two batches of juvenile mussels were cultured in 1994. The first batch of juveniles (mean initial length = 0.29 ± 0.01 mm) yielded a mean survival rate of 3% to 112 days (Table 1, Fig. 4), with one container having 17% survival. The survival rate of juveniles to 93-100 days during the second batch (mean initial length = 0.40 ± 0.05 mm) was very low, less than 1% (Table 1, Fig. 4). The empty shells in the containers indicated that death occurred sometime between 14-20 days old, based on the number of fine growth lines. Neither substrate size nor depth had an effect on juvenile growth or survival.

Table 1. Effects of daily mean temperature on the growth and survival of juvenile rainbow mussels (*Villosa iris*) held in the culture systems at the Clinch River Power Plant in Carbo, Virginia.

Year	Number of days with mean temperature above 15°C	Initial length (mm) ± SD (n)	Final length (mm) ± SD (n)	Percent survival
1993	119	NA	2.10 ± 0.62 (18)	27.5
1994a	106	0.29 ± 0.01 (15)	1.81 ± 0.38 (19)	2.2
1994b	41	0.40 ± 0.05 (95)	0.42 ± 0.04 (10)	0.0
1995	79	0.25 ± 0.02 (20)	0.77 ± 0.08 (51)	19.1

The 1995 culture trial was sampled at intervals to determine whether mortality occurred early or was evenly distributed during the approximately 100-day culture trial. The overall mean survival rate to 94 days was 21.1% for the four substrate treatments combined (Table 1, Fig. 4). Juvenile survival to 30 days was 40.8% in the fine sediment, the only sediment size included in the interval sampling. The sample at 74 days yielded a 17.3% survival rate in the fine substrate (both depths combined), based on the initial number of juveniles. When calculated from day 30 to day 74, the juveniles exhibited a

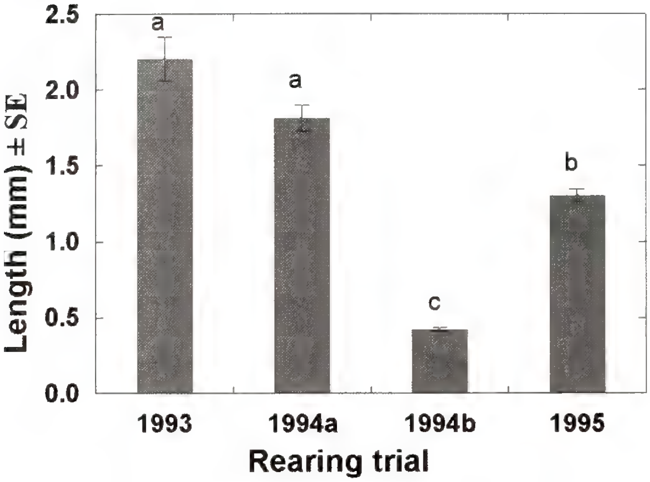

Figure 5. Mean shell lengths of juvenile rainbow mussels (*Villosa iris*) at the end of each rearing trial. All trials were conducted in the same culture system, but differed in years and time of initiation. Error bars are ±1 SE and those with different letters are statistically different at the α = 0.05 level.

42.4% survival rate during this interval. There was no measurable mortality between day 74 and day 94, resulting in a mean survival rate of 21.1%. The final survival rate for the 1995 trial was not significantly different from that of the 1993 trial (Fig. 4). However, the growth rate of juveniles during the 1995 trial (mean initial length = 0.25 ± 0.02 mm) was lower than during the 1993 trial (Table 1, Fig. 5). Substrate depth and substrate size had no effect on growth or survival.

Effects of stocking density

Stocking density had no effect on the survival or growth of the juveniles, but good growth rates were obtained in the stocking density laboratory experiment, conducted in a recirculating system maintained at a temperature of 23-27°C. Juveniles (mean initial length = 0.453 ± 0.028 mm) held in this system for 60 days reached sizes ranging from 1.5-2.0 mm in length. This growth was similar to that of juveniles in the natural river water culture system under the best-performing trials. Total hardness (~240) was higher and pH (~7.2) was lower than in the natural river water system or the temperature experiment system.

Temperature effects

During the laboratory experiment to assess the effects of temperature, definite trends in survival and growth of juveniles were evident among treatments. Initial length of juveniles was 0.274 ± 0.022 mm. At day 30, mean survival and length were 79.3% and 0.40 mm, 71% and 0.45 mm, and 49.7% and 0.50 mm in the 12°C, 18°C, and 25°C streams, respectively. No juveniles survived to 60 days in the 25°C treatment. Mean survival rates to day 60 were significantly different ($p < 0.05$) for the 12°C and 18°C streams; 76% and 33%, respectively. Mean lengths at day 60 also were statistically different ($p < 0.05$); 0.36 mm and 0.44 mm for the 12°C and 18°C treatments, respectively. Shell lengths of juveniles in the 12°C stream after 60 days were not statistically different from those at the start of the experiment.

In order to evaluate growth rates among experiments, within this study and for comparison to other studies, a method of normalization was used. The growth achieved during each experiment (1993, 1994, and 1995 culture trials, temperature experiment, and stocking density experiment) was calculated on the basis of the number of degree-days above 15°C experienced by the juveniles (Table 2, Fig. 6). Since water temperature significantly affected the growth rate of juvenile mussels, a comparison of degree-days was a suitable method for evaluating results. Once the data from each experiment were normalized on this degree-day basis, a regression was calculated to estimate the rate of growth of juvenile rainbow mussels and predicted lengths were calculated (Table 2). The regression equation with statistical outliers removed was:

$$\text{Length (mm)} = 0.00081 * \text{Degree-days} + 0.347.$$

The slope of the resulting regression was significantly different from 0 ($p < 0.001$) and had an R^2 value of 0.88, indi-

Table 2. Relationship of degree-days above 15°C to predicted and measured mean length (mm) for juvenile rainbow mussels (*Villosa iris*).

Degree-days above 15°C	Mean length (mm)	Predicted length (mm)	Source
90	0.45	0.42	this study
169	0.42	0.48	this study
180	0.44	0.49	this study
180	0.41	0.49	Gatenby 1994
240	0.66	0.54	Gatenby 1994
258	0.59	0.56	this study
300	0.50	0.59	this study
400	0.81	0.67	Gatenby 1994
465	0.76	0.72	this study
468	0.77	0.73	this study
480	0.69	0.74	Gatenby 1994
560	1.85	0.80	O'Beirn et al. 1998
660	1.07	0.88	Gatenby 1994
760	1.81	0.96	this study
800	1.01	0.99	Gatenby 1994
819	2.17	1.01	this study
1088	2.97	1.23	Gatenby 1994
1120	1.23	1.25	Gatenby 1994

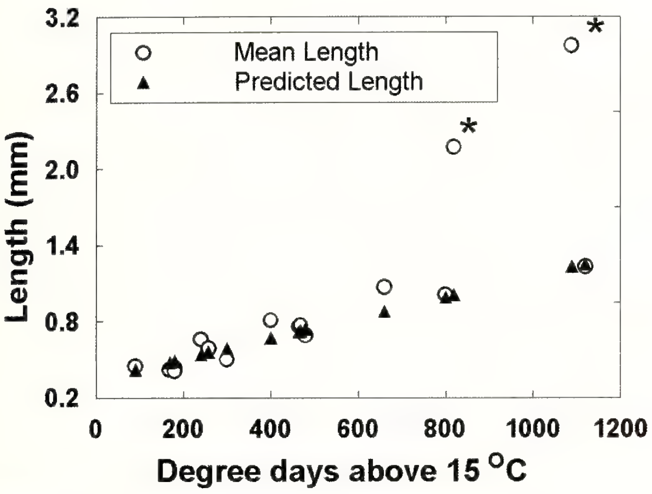

Figure 6. Plot of mean measured lengths and predicted lengths of juvenile rainbow mussels (*Villosa iris*) after culture at the given number of degree-days above 15°C. The regression equation used to calculate the predicted lengths was L(mm) = $0.809 \times 10^{-3} \times$ degree-days + 0.347. Points marked with an asterisk were determined to be statistical outliers.

cating that degree-days above 15°C accounted for most of the difference in the growth rate among experiments. Data from 1993 and the first batch in 1994 were omitted for calculating the above regression because they were determined to be statistical outliers, based on residuals analysis, relative to the other trials. Growth during the experiments in 1993 and first batch in 1994 was more than double the predicted value, with juveniles reaching a length of 2.22 mm and 1.81 mm vs. a predicted length of 1.01 mm and 0.82 mm, respectively. Growth data from two other previously published reports more closely matched the outliers from our study. When regression was performed on the growth per degree-days from the two statistical outliers of our study, the high growth trial of Gatenby (1994), and O'Beirn *et al.* (1998), the regression equation was:

$$\text{Length (mm)} = 0.002 * \text{degree-days} + 0.38,$$

with an R^2 of 0.83 and $p < 0.10$.

DISCUSSION

The substrate characteristics of the culture system with natural river water had little effect on the growth and survival of juvenile rainbow mussels. Substrate depth did not affect survival or growth during any of the trials. Juvenile rainbow mussels are pedal feeders and depend on suitable food being present in the substrate for their nutrition (Yeager *et al.* 1994). Additionally, previous studies reported that juvenile mussels burrow into the substrate to depths great enough to avoid interaction with currents in the water column, as much as 8 cm below the substrate surface (Neves and Widlak 1987, Yeager *et al.* 1994). However, juveniles did not burrow deeply into the substrate while in the present culture system. After 30 days, over 95% of the juveniles were still at the surface of the provided substrate, but a 1-5 mm flocculent layer of fine particulate material accumulated in the dishes. The juveniles were residing in the flocculent layer. As the juveniles aged, a higher percentage were found burrowed into the sediment, but only 23% had burrowed into the substrate by 95 days of age. This result differs from the findings of Yeager *et al.* (1994) in which juveniles were not found in the top 1-2 mm of substrate, a strategy speculated to prevent resuspension in flowing water. The fact that juveniles remained at the surface of the substrate in the present culture system suggests that either the surface conditions provided by our culture system were favorable for the juveniles, or the habitat conditions of the consolidated, lower interstitial environment were unfavorable. Because juvenile rainbow mussels initially obtain food through pedal-feeding (Yeager *et al.* 1994), it is reasonable to expect that a layer of fine particulate material from natural river water would provide nutritional resources. Neves and Widlak (1987) reported that juveniles often inhabit depositional areas in streams that would contain similar fine particulate materials. Therefore, the presence of particulate material in culture systems is beneficial to juvenile mussels (O'Beirn *et al.* 1998).

The lack of an effect of substrate size on the rearing of juvenile mussels indicated that the mussels utilized the very surface layer of fine particulate material when young. Substrate size had no effect on juvenile survival, indicating that the juveniles were capable of finding suitable habitat in both fine sediment (<120 μm) and sand (120-600 μm). Additionally, the effect of substrate size on growth was minimal, being significant only during the first trial in which juveniles in the fine substrate grew slightly faster. The slightly greater growth rate in the fine substrate may have been due to the presence of a greater density of Asian clams (*Corbicula fluminea* Müller, 1774) in the larger substrate. The average density of 8 clams per dish, or 1 clam per 5 cm^2, in the large substrate may have been high enough to negatively impact the juvenile mussels. Yeager *et al.* (2000) reported that Asian clam densities as low as 1 clam per 10 cm^2 caused 100% mortality of juvenile rainbow mussels, and densities of 1 clam per 40 cm^2 caused 93% mortality. The fine substrate treatment had an average density of 2 clams per dish, or 1 clam per 21 cm^2. Perhaps pediveligers or small juveniles did not get sieved out of the larger substrate fraction but were effectively removed from the smaller substrate size. Another possible explanation for the greater number of Asian clams in the coarse substrate was that these animals were brought in with the river water and survived better in the coarse substrate than in the fine substrate. The overall lack of an effect of substrate size was consistent with the finding that juveniles predominantly exploited the loose layer of fine particulates at the surface. While substrate size did not have a significant influence on juvenile mussel survival or growth, Buddensiek (1995) and O'Beirn *et al.* (1998) showed that the presence of some substrate improves growth and survival rates of juvenile mussels.

The time of year when juveniles were introduced into the culture system strongly influenced the success of the rearing trials. The differences in juvenile growth among the three trials correlated well with the timing of placement of juvenile mussels in the troughs. The later the juveniles were introduced into the culture system, the less they grew. Buddensiek (1995) reported that the sizes of juveniles at the start of winter played a large role in determining mortality, with all animals below a minimum size dying. The results of our study support the hypothesis that juveniles must achieve some minimum size to overwinter successfully (Hanlon 2000). With the one exception of the early 1994 trial, the mean survival rate was positively correlated with the mean growth.

Differences in water temperature explained much of the variation in growth of juvenile mussels. Based on our data from the laboratory experiment, we determined that juvenile rainbow mussels grew very little at water temperatures below 15°C. Therefore, temperature effects were evaluated on the basis of cumulative degree-days above 15°C. The strength of the relationship between cumulative degree-days and shell length in both the natural river water system and the laboratory experiment indicated that temperature was the principal factor affecting growth rate. The same quantitative relationship between cumulative degree-days and size effectively predicted growth in juvenile rainbow mussels in Gatenby (1994) as well. However, three trials did not fit this relationship. Both the 1993 and first 1994 trials of this study, an algae plus sediment trial of Gatenby (1994), and a recirculating system study (O'Beirn *et al.* 1998) yielded greater than double the growth predicted based on cumulative degree-days alone. Such a significant increase in growth suggests that once juveniles reach a certain size, their growth rate increases because of better organ development and filtration efficiencies. Juveniles in these trials may have gotten a faster growth start during the first few weeks due to seasonal differences in environmental conditions at the start of culture trials, allowing them to become more effective at obtaining nutrition throughout the rest of the experiments.

When compared to previous studies on the culture of juvenile mussels, the results of our natural river water troughs were mixed. The poor survival rates in the 1994 trial were problematic because the cause of mortality was unknown. Late introduction into the culture system may have doomed the second batch of 1994 to failure, because temperatures were low enough to prohibit significant growth of the juveniles. Survival rates to approximately 90 days for juvenile rainbow mussels in other studies range from 2.7% to 36%, with a mean survival of about 25-30% for successful trials (Gatenby *et al.* 1996, O'Beirn *et al.* 1998). Therefore, the survival rates of approximately 20% obtained in the 1993, 1995, and the stocking density trials were similar to those of previously published studies. When normalized by cumulative degree-days, the highest growth rates in this study did not exceed those of one trial of Gatenby *et al.* (1996) or O'Beirn *et al.* (1998), but were significantly higher than most other growth rates in laboratory experiments. The reasons for the disparate growth rates likely relate to food availability and suitability in the systems. Both Gatenby *et al.* (1996) and O'Beirn *et al.* (1998) fed the juveniles an algal diet to supplement the food available in the sediment. The culture system used in our study depended on natural river water and interstitial biota and detritus to provide nutrition for the juveniles. While the natural food supply should be nutritionally adequate, the supplemented diets may have provided additional energy for growth. The presence of sediment in culture systems has proven to be beneficial to juvenile mussels (Hudson and Isom 1984, Buddensiek 1995, Gatenby *et al.* 1996, O'Beirn *et al.* 1998). However, there seems to be no significant difference between the suitability of fine silt and sand, or substrate layers deeper than 5 mm. Newly transformed juvenile mussels pedal-feed during the first stage of life (Yeager *et al.* 1994, Gatenby *et al.* 1996). The presence of a substratum is believed to provide a medium for the juveniles to feed effectively, position themselves, and avoid disturbance by turbulence or vibrations (O'Beirn *et al.* 1998). The hypothesis that sediment is beneficial due to physical protection from turbulence was supported in our study; we observed higher survival rates in culture dishes that showed little evidence of disturbance of the substratum surface. Because juvenile mussels were usually found in the loose layer of sediment at the surface, physical disturbances would have caused stress to the mussels as they tried to reposition in the substrate. O'Beirn *et al.* (1998) hypothesize that similar stresses on juveniles during the process of sampling and measuring caused reductions in growth over time.

The culture system used in our study, supplied with Clinch River water, shows potential for effective use in a mussel propagation program. Recent work at the Buller Fish Hatchery in Marion, Virginia, using South Fork Holston River water supports the value of using natural river water in culture systems. High survival and growth rates with juveniles of several species of mussels have been reported (Mike Pinder, pers. comm.). One advantage of this type of culture facility is the relatively low level of maintenance that is required. Culture systems with natural river water provide sufficient sediment for the juveniles to use for feeding and positioning. Water chemistry also is not a concern, because these systems employ flow-through water from a source that is known to support numerous species of mussels.

However, this type of river-side culture system has disadvantages. The most significant drawback to using this system is the strong influence of season on the growth and survival of juvenile mussels. If the culturist is unable to produce adequate numbers of juveniles by early summer, the total production for the year may be jeopardized, with low over-winter survival. The use of water heaters and retention ponds can help to minimize this problem, but heating the large amounts of water used in a flow-through system is impractical. Using natural river water allows pests or predators to invade the culture system and affect the juvenile mussels. While some filtration can reduce this threat, it cannot be eliminated. Finally, the culture system is susceptible to water quality insults or toxicants that might enter through the river water in the event of a spill or other catastrophic event. The only measure that can mitigate that risk is the ability to shut off flow for some time and shift the system to a recirculating culture system. Even with these few disadvantages, a culture system using natural river water is useful for a mussel propagation program due to the low operational costs, avoidance of supplemental feeding, and high potential growth and survival rates of juvenile mussels.

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Integrating historical and functional data to examine feeding in gastropods*

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Abstract: This paper details a logical framework for integrating phylogenetic and functional approaches based on examining how structures and functions change during the course of evolution. Cladogenetic events lead to sharing of primitive or derived developmental trajectories that in turn can lead to shared, derived, adult phenotypic traits different from those found in more basal taxa. Change in adult phenotype can lead to changes in how structures perform mechanically. To document these transitions in function and infer the potential causes for those functional changes, I suggest the following steps: (1) Show a functional difference in different taxa; (2) Determine the polarity of the change and when in evolution the change happened based on sampling more taxa in the context of existing phylogenies; (3) Determine the morphological characters that have changes concordant with change in function; (4) Model or use experimental techniques to determine which morphological characters are causal in the change in function. I apply this scheme to a case study of a change from flexoglossate to stereoglossate function in the gastropod radula.

Key words: cladogenesis, phenotype change in function, radula

The gastropod feeding system has the potential to be a model system for understanding and synthesizing the underlying phylogenetic, developmental, and ecological controls on diversity and diversification of form and function. Modifications of the gastropod feeding apparatus have long been recognized as a crucial feature in gastropod diversification. The diversity of methods for capturing food and food sources among gastropods is extraordinary—as diverse as any other major lineage of animals. Gastropods use their feeding apparatuses as pincers to hold captured prey; scissors to cut algal blades; powerful gouging devices to remove hard algae; scrapers and sweepers to collect detritus; injectors of toxins to paralyze prey; and suction devices to remove prey from tubes or other hard to reach areas, as well as many other uses.

The anatomy and function of gastropod feeding systems are complex with many associations between the major subsystems (radula, odontophore, jaw, muscle, nervous control) that function during feeding. Despite complex interrelations, the sub-systems that make up the feeding apparatus are clearly discrete and easily recognizable. It is therefore relatively easy to determine whether or not changes in subsystems or interactions between sub-systems lead to functional novelty. Taken together, diversity in feeding mode and anatomical complexity provide ample variation and provide the potential of covariation between feeding mode and structural/functional changes. In addition, the gastropod feeding system is already a model system for neurobiologists who are interested in nervous control of very fast, repetitive muscle motions. Because of this, more is understood about neural control of muscles used in feeding than in most other molluscan anatomical systems.

Despite the potential of the gastropod feeding system as a model system, a synthetic approach to structural and functional transformations has generally been lacking (but see Graham 1973 for one attempt). Lack of such a synthesis is surprising given the careful examination and detailed single-species accounts of anatomy and functional morphology of these systems (Huxley 1853, Geddes 1879, Amaudrut 1898, Woodward 1901, Herrick 1906, Crofts 1929, Carriker 1943, Starmühlner 1952, Hubendick 1956, Fretter and Graham 1962, Fretter 1965, Graham 1965, Morris and Hickman 1981, Hickman and Morris 1985).

Two problems have prevented a more cohesive synthesis of anatomical, functional, and phylogenetic datasets. One problem has been a lack of robust phylogenetic hypotheses in which to contextualize the functional diversity. Recently, such hypotheses have become available for many molluscan clades (*e.g.*, Ponder and Lindberg 1997). The second and perhaps larger problem has been lack of a general model or approach in which to conceptualize the dynamics of change in the feeding system. Such a model should provide the means to test that changes during the course of evolution have led to new structural modifications that in turn have led to new functional modifications and to test how these relate to past and present ecologies. The intent of this paper is to provide a general model to approach the question of feeding modifications during evolution using a framework similar to that developed by Lauder (1990) and to provide a case study on the shift from flexoglossy to stereoglossy (Guralnick and Smith 1999) that uses elements of this general model.

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A GENERAL MODEL OF CAUSALITIES

The main goal of this paper is to provide a simple, generalized model of causalities, focusing on macroevolutionary changes and the outcomes of those changes in regard to development, adult phenotype, and function. The relationship between phylogenetic, developmental, and functional change can be viewed as a series of causalities starting with a phylogeny (Fig. 1). For example, the phylogeny represented in Figure 1 depicts two speciation events that have created separate historical units. The first event separated lineage A from the lineage that eventually splits again to form B and C (as labeled in Fig. 1).

In this example, these events have led to a major change in the development of a particular trait between lineage A and lineages B and C, shown in the first box labeled "Developmental change." The developmental change of that trait (*e.g.*, the radula or buccal muscle system) is represented by an arrow that maps out the shape of the trait as it develops. The spheres represent variation among individuals. The rearrangements of developmental pathways leading to novel trajectories in the descendent lineages B and C ultimately lead to novel adult phenotypes as shown in the box labeled "Adult phenotypic change." In this box as well, spheres represent variation of that trait within the species.

When the shape or position of a trait changes, the function of that trait may be different depending on the system as a whole. The functioning of parts may display a range of variation depending on nervous controls and the action of other parts that make up the system. This variation is represented by arrows in the box labeled "Functional change." Each lineage may display a different range of possible kinematics—with similar shapes more likely to function in the same way. An organism's parts function throughout the life of an organism. Therefore they may be in use at all points along the developmental trajectory.

The foregoing discussion features elements inherent to the organism to describe functional change. Changes in structure and function along lineages can feed back to the beginning of this hierarchy and affect later potential for speciation (*e.g.*, key innovations). This is represented by the arrow from the box labeled "Functional change" to the bottom of the diagram.

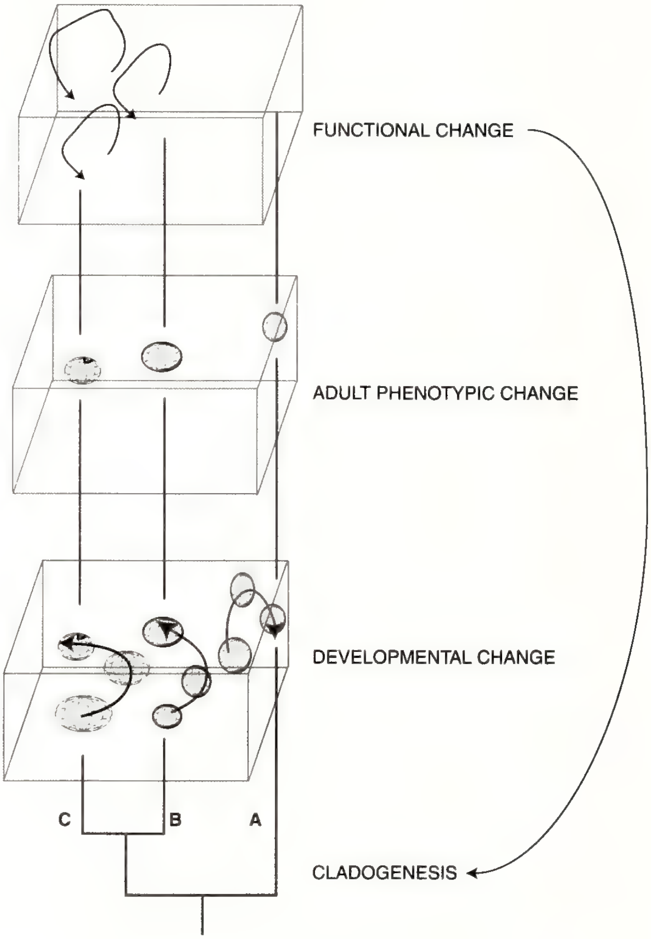

Figure 1. A macroevolutionary view of structural and functional causalities. Speciation leads to independent lineages that may share primitive or derived developmental pathways for any one phenotypic trait. Different developmental pathways will lead to different adult phenotypes and ultimately different ranges of functions. In the example shown, lineages B and C share a derived developmental pathway leading to a different adult morphology and ultimately different kinematics for that particular phenotypic trait. The boxes labeled Developmental change, Adult phenotypic change, and Functional change represent constructed spaces based on measurements of structures made during development, adulthood, and during functioning. The spheres at different points in the measured spaces represent intraspecific variation. The shapes in the box for functional change represent different possible functions a single structure might perform.

In most cases, many traits are involved in making a fully functioning system. In the case of gastropod feeding, four primary systems are involved—the radula, odontophore, muscle, and nervous system. The radula and its underlying subradular membrane, for example, can be viewed as an aggregate of traits at a structural level because they are produced by different organs, and as a unit or a single trait at another because radula and membrane are moved as a unit during feeding. Each system may ultimately show similar or different evolutionary trajectories.

A SYNTHETIC APPROACH TO UNDERSTANDING STRUCTURE-FUNCTION DYNAMICS

Work especially by Lauder (1990) has documented how to examine structural and functional changes in an evolutionary context. The discussion here generally follows Lauder's (1990) work, with minor modifications given sampling problems in our knowledge of gastropod feeding structure and function.

One way to document changes in gastropod feeding is first to recognize functional shifts and work back from those shifts to structure and phylogeny—basically working backwards from the top of Figure 1 to the bottom. In the example that follows I skip developmental changes, although studying that level as it relates to structure and function will likely prove fruitful. The following case study involves a major change in the way the feeding system functions: the shift from the radular teeth being flexed laterally during feeding to teeth remaining fixed or vice versa.

Once functional shifts have been documented, the next step is to map those shifts onto a known phylogeny. Thus, the functional shifts themselves become characters to include in the phylogeny. The goal is to pinpoint when exactly during the course of evolution the shift from one function to the other actually occurred. This requires adequate sampling of lineages because the functional shift most likely did not arise in the first taxon sampled but instead originated earlier in the diversification of lineages.

Pinpointing when and where the change occurred during evolution is a crucial first step in documenting *how* the change occurred. For example, if the plesiomorphic condition for gastropods is to flex the radula, and that state has changed in only one clade of gastropods, and all members of that clade share that characteristic, it is reasonable to assume that some of the structural modifications that led to the derived function also arose at the same point in phylogeny (*i.e.*, in the origin of that clade). Other modifications of the system in more derived members within that clade may have important kinematic outcomes, but they are unlikely to be causal in that particular change. It is also possible that some of the machinery that led to novel function was modified in more basal lineages and co-opted for that function. However, some structural or behavioral change must have occurred simultaneously with change in function. The structural character changes in that transform concordant with functional changes are the ones to examine as potentially causal in that functional change.

The concordance between structural and functional changes on a phylogeny are not necessarily causal but instead may be merely correlated. Thus, some structural characters may have changed state but have no direct impact on functional change, while others may have transformed and have been directly causal in the functional change. The next step is to determine which character state changes have caused functional changes. This step requires no direct historical information, but instead involves either experimental or model-based approaches based on the physical properties of the system.

In a model-based approach, character state transformations might serve as parameters that can be tested based on the physical properties of the systems involved. For example, the change of position of a muscle insertion based on a change in character state from the phylogeny can be tested for impact on functional change in a computer simulation in which all the other components are kept the same. This change may not be great enough to explain the overall functional change and other parameters based on the actual structural transformations can be incorporated into the simulation.

To summarize this approach to understand the evolutionary and causal dynamics of structure and function: 1) Determine functional differences between taxa that are important or easy to recognize; 2) Determine the polarity of those changes and when exactly the change occurred during evolution of the larger group you are studying; 3) Determine the structural character state changes that occur concurrently with functional shifts; 4) These character transformations become candidates for being causally involved in functional change. The character state changes can become parameters in a model or an experiment that tests how function changed.

A CASE STUDY IN GASTROPOD FEEDING: FLEXOGLOSSY *VS.* STEREOGLOSSY

STEP 1: Delimit the functional differences

Radular teeth can either flex laterally outwards and then sweep inwards (the *flexoglossate* condition) or the teeth can remain fixed (the *stereoglossate* condition) during the feeding stroke (Salvini-Plawen 1988, Ponder and Lindberg 1997).

STEP 2: Determine polarity of change and where the change in functioning occurred

The polarity of the changes between flexoglossate and stereoglossate has been a point of some debate (Guralnick and Smith 1999), but based on both written descriptions and high-speed video of feeding mechanics in the polyplacophorans (Jüch and Boekschoten,1980, Padilla, pers. comm.), it appears that the plesiomorphic (primitive) state in gastropods is flexoglossy. The only apparent change in state (from flexoglossy to stereoglossy) probably occurred at the base of the Patellogastropoda, the most basal clade of gastropods. Despite imperfect taxon sampling of the Patellogastropoda due to many deep-sea groups in which feeding function is difficult to study, the state change from flexoglossy to stereoglossy is unambiguously placed at the base of the patellogastropod tree (Ponder and Lindberg 1997).

STEP 3. Determine structural changes that happen concordant with functional change

Based on a preliminary phylogenetic analysis, Guralnick and Smith (1999) documented that the major changes that occurred concordant with functional change were not in the radula of the Patellogastropoda, but in the odontophores. There are no radular morphological changes coded that unambiguously change concordant with functional change. In the odontophore, however, five state changes occurred at the same time as the shift from flexoglossate to stereoglossate. Those five changes were: (1) A shift to odontophores not linked by connective tissue; (2) Shape of medial odontophores shift from same height from anterior to posterior to being highly elongate anteriorly but not posteriorly; (3) A shift to dorsal odontophores being unambiguously present; (4) A shift to the radula being supported by strong and large dorsal odontophores instead of the medial odontophores; and (5) A shift to the dorsal-lateral odontophores being unambiguously dorsal to the medial odontophores.

STEP 4: Determine a model or conduct an experiment based on the character state changes

Guralnick and Smith (1999) proposed a very general model with three parameters that must be met for a system to be flexoglossate. The three parameters are: (1) Some sturdy anterior structure must be present that will support the radular membrane as it is pulled over the bending plane; (2) The radula must be folded about a longitudinal axis and the subradular membrane must be closely associated with the underlying structural supports prior to reaching the location of the bending plane; and (3) The radular apparatus must be partially or fully flattened as it passes over the bending plane. Guralnick and Smith (1999) showed, using very simple calculations based on the shape and position of odontophoral cartilages and radula, that under these conditions partial or full flattening of the radula can occur passively due to the shape of the anterior medial cartilages.

The main way in which patellogastropods differ from polyplacophorans and more derived gastropods is lack of a close association of the radular apparatus and underlying medial ondontophores near the bending plane. Dissociating the subradular membrane from the supporting structure for most of the length of the buccal mass means that the radular teeth in patellogastropods are never in position to rotate around the edge of the ondontophores posterior to the bending plane. Further, the teeth are never forced into the groove between the ondontophores and thus are also not infolded prior to the bending plane. Thus the patellogastropods do not fulfill parameter two of the model above and therefore do not flex during feeding.

CONCLUSIONS

The conceptual framework described here for linking phylogeny and function is based on the fact that cladogenesis and concordant character changes in anatomy can ultimately lead to novel function of those anatomical units (Fig. 1). In the case study, the major functional transition from flexoglossy to stereoglossy is shown to occur in Patellogastopoda. Characters from the radula and odontophoral cartilage systems were examined on the most parsimonious tree and the state transformations concordant with the functional shift were examined as potentially causal. A simple, general model that takes into account the physical properties of the radula and cartilage system is presented for the plesiomorphic (primitive) state of flexoglossy. In this case, dissociation of the radula from the cartilages has led to lack of flexure of the radula as it passes over the bending plane

Integrative approaches linking structure, function, phylogeny, and ecology as it relates to feeding in gastropods has not been one of the major thrusts of malacological endeavor over the last twenty years. However, the timing for such a synthesis could not be better because of improved molluscan phylogenies, especially within the major classes (*e.g.*, Ponder and Lindberg 1997 for Gastropoda). Given the large body of studies of single-taxon feeding systems, much of the core data is already available in the literature and awaits synthesis. From there, it is easy to develop targeted sampling of groups to answer some fundamental and interesting questions about how feeding evolved within the Mollusca. As Ponder and Lindberg (1997) and Guralnick and Smith (1999) have pointed out, feeding innovation is an important issue in molluscan evolution, but knowing how molluscs feed is as important as knowing on what they feed on. To understand the past and current ecological conditions and how molluscs fit into those ecologies will depend on documenting multiple lines of evidence about feeding, from structural/kinematic issues to “how it is eaten” and “what is eaten.”

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The biology and conservation of freshwater gastropods: Introduction to the symposium

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Key words: Freshwater snails, Ecology, Conservation

About 20 years ago I became interested in the cytogenetic evolution of pleurocerid snails, a group of organisms that includes the very symbol of the American Malacological Society. The family is quite diverse in North America; its 150 nominal species being divided into seven genera and twelve subgenera, its representatives often rising to the status of dominant grazers, especially in rivers and streams of the southeast. My initial idea was to sample at least one population from each of the higher pleurocerid taxa for a systematic study of the karyotype.

My plan was doomed, however, to run afoul of biological reality. The type of the subgenus *Strephobasis* Goodrich, 1928 is *Pleurocera* (*Strephobasis*) *curtum* (Haldeman 1841), a robust and heavily-shelled inhabitant of larger rivers in the Tennessee and Cumberland drainages. It has a reported range of Alabama, Tennessee, and Kentucky. But over a series of field trips in the mid-1980s I became increasingly frustrated in my efforts to discover a single extant population. I remember staring at the barren rock substrate of the Cumberland River in Russell County, Kentucky, USA, where the water had been chilled nearly to freezing by passage through the Lake Cumberland Dam, when the realization struck me that *P. curtum* is extinct.

In the last ten years I have repeatedly challenged my colleagues in malacology to locate a single living individual of this species, with no result. What makes the situation especially alarming is that this large and important element of the freshwater gastropod fauna of North America apparently became extinct without our notice. Reference to the *NatureServe Explorer* (http://www.natureserve.org), the most up-to-date database of which I am aware, returns a "heritage rank" of G2 for *Pleurocera curtum*, intermediate on a conservation scale of G1-G5.

In the late 1980s I developed a fresh research focus in the family Physidae, a group of organisms as different from the Pleuroceridae as can be imagined within the boundaries of the Gastropoda. Physids are typically small, thin-shelled, and rapidly-growing. The population with which Amy Wethington and I began our studies reached high densities in a eutrophic pond in the park about five blocks from my home. Physids are marvelous experimental organisms, laying hundreds of eggs in small cups of stagnant water, completing their life cycle in weeks, rather than the years required by pleurocerids. I think that physids may become the "fruit fly of malacology," so readily adaptable are they to laboratory culture.

Yet in some sense we were, and perhaps still are, as ignorant of that common physid snail inhabiting the pond in my suburban neighborhood as we are of *Pleurocera curta*, the illusive denizen of select, free-flowing rivers of the American interior. For we did not know the specific identity of our local populations until 1995, when studies of reproductive isolation finally confirmed it to be *Physa acuta*, a species first described in France but (probably) native to North America, long identified here by other Latin binomina of more recent coinage.

Freshwater gastropods are a polyglot fauna of pulmonates and "prosobranchs," annuals and perennials, grazers and filter-feeders, hermaphrodites and gonochorists, egg layers and live bearers, selfers, outcrossers, and parthenogens united by a patina of familiarity and a core of ignorance. As a small contribution toward the amelioration of this situation, in 2002 I organized a symposium entitled "The Biology and Conservation of Freshwater Gastropods" for the American Malacological Society meeting in Charleston, South Carolina, USA. And I was most pleased that Amy Wethington agreed to arrange a special session entitled "Pulmonates in the Laboratory" as a companion to the main symposium. Together these two sessions yielded the 12 papers published here, covering topics as diverse as intraspecific competition, predation, ecological succession, population divergence, speciation, morphology, and behavior. Our intent was to show freshwater gastropods for the powerful research tools they can be, outline the known, and point the way to the unknown. Our hope is that any increment in our knowledge of this fauna, however modest, will yield an increase in appreciation, and appreciation to conservation. We cannot afford delay.

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Intraspecific competition and development of size structure in the invasive snail *Potamopyrgus antipodarum* (Gray, 1853*)

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Abstract: The clonal, invasive New Zealand mudsnail *Potamopyrgus antipodarum* is rapidly spreading throughout rivers and estuaries of the western USA, where its densities can exceed 300,000/m^2. Although very little is known about its ecology in the western USA, *P. antipodarum* can compete with native invertebrates and appears to have altered primary and secondary production in several western aquatic systems. There are no data published on intraspecific competition and density dependence for this species in the western USA. Populations contain a wide range of sizes; mechanisms responsible for this size distribution are unknown. We conducted laboratory growth experiments on *P. antipodarum* to determine if intraspecific competition would cause shifts in size hierarchy and decrease growth rates. We used the Gini coefficient (G) to evaluate size hierarchy shifts. Our results showed that asymmetric competition resulted in a strong size hierarchy and that an increase in snail densities caused a decrease in growth. These results suggest that size hierarchies of populations of *P. antipodarum* might not be entirely based on age class and that asymmetric competition may affect its ecology.

Key words: Intraspecific competition, size hierarchy, *Potamopyrgus antipodarum*

The invasive New Zealand mudsnail *Potamopyrgus antipodarum* (Gray, 1853) (Hydrobiidae) (Fig. 1) is rapidly spreading throughout river systems in the western USA. It first became established in the mid-Snake River, Idaho in the mid-1980s (Bowler 1991) and has since spread to most major western river systems (Fig. 2). *Potamopyrgus antipodarum* is native to New Zealand and is well established throughout many rivers and estuaries in Australia, Europe, and Asia.

In the western USA, densities of this parthenogenic livebearer can exceed 300,000/m^2 and have been reported as high as 750,000/m^2 in a tributary of the Snake River in Yellowstone National Park (Robert Hall and Mark Dybdahl, pers. comm.). To date, limited research on the ecological impacts of *P. antipodarum* on western USA aquatic ecosystems has been published. We demonstrated that under field and laboratory conditions, *P. antipodarum* caused decreased growth in the threatened Bliss Rapids snail, *Taylorconcha serpenticola* Herschler, Frest, Johannes, Bowler, and Thompson, 1994 and decreased growth in conspecifics through increased intraspecific competition (Richards and Shinn 2001, 2002). Chelsea Cada and Billie Kerans (pers. comm.) have observed decreased densities of *Brachycentrus* sp. (Trichoptera: Brachycentridae), *Baetis* sp. (Ephemeroptera: Baetidae), and chironomids (Diptera), which they attribute to interference and exploitative competition with *P. antipodarum*. They also have observed decreased biomass of periphyton in sections of their study site containing *P. antipodarum*. Robert Hall and Mark Dybdahl (pers. comm.) found more than 70% of the primary production in several rivers in Yellowstone National Park has been diverted through *P. antipodarum*. Their data show that *P. antipodarum* consumed up to 100% of the algal production and that algal growth rates were slower with increased biomass of *P. antipodarum*, which suggests to them that *P. antipodarum* was consuming high-turnover algal taxa.

Potamopyrgus antipodarum now often comprises 85% to 95% of the biomass and abundance of the invertebrate assemblages in many rivers in the western USA (Bowler 1991, Richards *et al.* 2001, Chelsea Cada and Billie Kerans, pers. comm., Joseph Shannon, pers. comm., Dawne Becker, pers. comm.). Not only is *P. antipodarum* a highly invasive species, but it has the potential to affect populations of native species and alter ecosystem function. Because *P. antipodarum* can often reach extremely high densities and, under certain conditions, appears to be capable of affecting whole ecosystems, understanding its population dynamics becomes critical if we are to control its spread and limit its impact.

Intraspecific competition may be an important regulator of populations of *Potamopyrgus antipodarum* and could influence population dynamics, including individual growth

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* From the symposium "The Biology and Conservation of Freshwater Gastropods" presented at the annual meeting of the American Malacological Society, held 3-7 August 2002 in Charleston, South Carolina, USA.

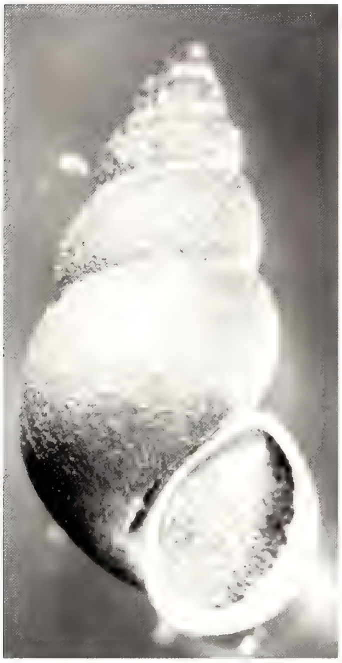

Figure 1. Preserved specimen of *Potamopyrgus antipodarum* with operculum attached, 5 mm long. Photo courtesy of Dr. Daniel L. Gustafson, Montana State University, Bozeman, Montana, USA.

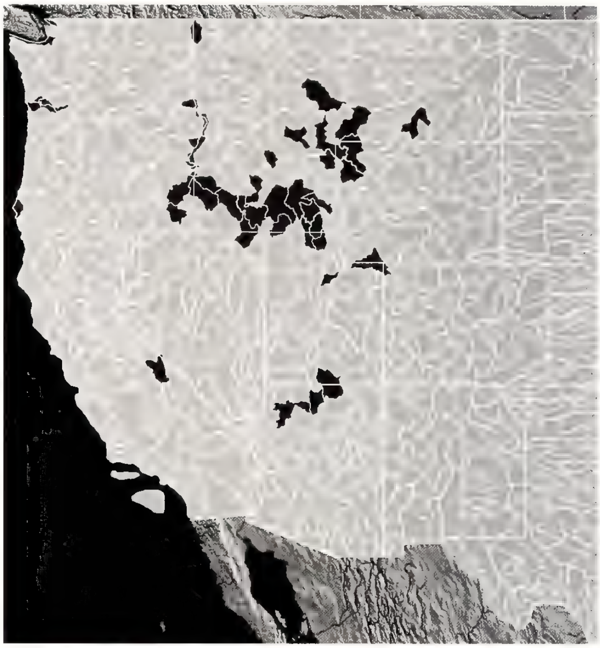

Figure 2. Hydrological units (HUC's) reported to be infested by *Potamopyrgus antipodarum* in the western USA. *Potamopyrgus antipodarum* has also been confirmed in Thunder Bay, Lake Superior and Lake Ontario, USA. (Richards *et al.* 2003).

rates. Size structures of populations of *P. antipodarum* vary in the western USA; sizes of individuals present at any one time may range from 0.5-1 mm newborns to 4-5+ mm adults of unknown ages. Because *P. antipodarum* is a clonal species, some researchers have attempted to use size classes to infer age structure, based on the assumption that genetically identical *P. antipodarum* within a population should grow at approximately the same rate, as compared with sexual snails with more genetic variability. This assumption may be inappropriate because "asymmetric" or "one-sided" intraspecific competition could influence the size hierarchy of a population (Begon 1984, Weiner 1990, Begon *et al.* 1996), even in a clonal species. Asymmetric competition occurs when larger individuals are able to obtain more resources than smaller individuals and thus suppress the growth of the smaller individuals (Weiner 1986, Begon *et al.* 1996). Size hierarchies induced by asymmetric competition have been reported for many organisms (Obeid *et al.* 1967, Ross and Harper 1972, Branch 1975, Uchmanski 1985, Gribbin and Thompson 1990, Adams and Tschinkel 1995, Geffen 1996).

The ecological and evolutionary significance of competition induced-size hierarchies are numerous (Begon *et al.* 1996). For example, because asymmetric competition suppresses growth in smaller individuals it could cause density-dependent mortality or decreased fitness in smaller individuals (Rubenstein 1981, Weiner 1985, Begon *et al.* 1996). Correlations between fitness (fecundity) and size have been well studied (Begon *et al.* 1996, McPeek and Peckarsky 1998, Taylor *et al.* 1998). We have demonstrated that larger individuals of *Potamopyrgus antipodarum* produce more offspring (Richards *et al.* 2000) and survive desiccation longer than do smaller *P. antipodarum* (Richards *et al.* in press), both of which may have ecological consequences. Thus, understanding the cause of size hierarchies in populations of *P. antipodarum* becomes important.

For this study, we conducted growth experiments on *Potamopyrgus antipodarum* in the laboratory to determine if intraspecific competition for limited food resources would cause shifts in the size hierarchy of populations. Because results from laboratory experiments are usually less variable and, therefore, more precise than are field experiments (Miller 1986), we chose to conduct laboratory experiments. This allowed us to control other variables that could have contributed to differences in growth. Also, it is extremely difficult and costly to conduct exclosure experiments with *P. antipodarum* at our field sites in the Snake River because of the sheer numbers of less than 1.5 mm newborn snails usually present, which can pass through any mesh large enough (1mm) to allow for flows that provide gas exchange and removal of metabolic waste.

MATERIALS AND METHODS

Experimental methods

We stocked individually measured *Potamopyrgus antipodarum* with shell lengths between 2.00 to 2.10 mm in double open-ended glass tubes (inner surface area 22.0 cm^2) at 1-snail/tube (20 replications) and 10-snails/tube (20 replications). We covered both tube ends with 1mm nylon mesh to allow passage of dissolved gases, including metabolic wastes. We have used this experimental design in numerous growth and competition studies and have found it to provide ample flow and gas exchange, even using snail species such as the oxygen demanding Bliss Rapids snail *Taylorconcha serpenticola* (David Richards, pers. comm.).

Each snail was individually color-coded by application of a small drop of various colors of acrylic fingernail paint to its apex. Stocked tubes were randomly placed in plastic test tube racks and submerged into a 605.66-liter (160-gallon) freshwater aquarium (water temperature 20°C). We placed snails into clean tubes with new mesh weekly to prevent establishment of periphyton in the tubes. Once per week, 1 mg of commercial *Spirulina* sp. dissolved in 5 ml of water was injected into each tube through the nylon mesh using a hypodermic syringe. Because *Spirulina* sp. is a poor food resource for most snails, we were assured that all snails were food resource limited. After one month, we measured growth of individuals to the nearest 0.05 mm.

Statistical analysis

We compared growth rates (mm/month) of individuals from 1-snail/tube and 10-snails/tube. Various indices (metrics) for measuring and comparing variability, evenness, and size hierarchies are often used in ecological studies (Knox *et al.* 1989, Damgaard and Weiner 2000) including skewness, coefficient of variation, the Shannon-Weiner index (Zar 1999), and the Gini coefficient "G" (Dixon 2001). According to Weiner and Thomas (1986), size variability is best measured in terms of inequality (hierarchy) and of these metrics the Gini coefficient is considered to be the most relevant (Weiner and Solbrig 1984, Dixon *et al.* 1987, Knox *et al.* 1989). For this study, therefore we focused our analysis on the Gini coefficient, G.

Because we used all twenty of the individual growth rates of snails at 1-snail/tube in the calculation of the no competition G (N = 20 data points), it would have been less accurate to compare the with-competition G derived from 10- snails/tube (N = 10 data points). Therefore, to create with-competition G's derived from 20 data points, we randomly sampled an individual snail from each of the twenty 10-snails/tube 1000 times using a random sampling algorithm and then calculated 1000 with-competition G's. We then compared 95% confidence intervals for the 1000 with-competition G's with the single no-competition G. We also conducted a one-tailed t-test comparing differences in means between the no-competition G and the 1000 with-competition G's. We used SAS for Windows (SAS Institute 2001) for our t-test and the program "Gini" developed by Borkowski (2002) in SAS for Windows (SAS Institute 2001) for generation of G.

G is a summary statistic that measures size hierarchy (inequality) in a population. It ranges from 0 for populations with all individuals of equal size, to 1 where every individual except one has a size of zero. We used the G formula presented by Dixon (2001):

$$G = \frac{\sum_{i=1}^{n} (2i - n - 1)X_i}{(n - 1)\sum_{i=1}^{n} X_i}$$

Here n equals the number of individual snails and X_i is the size of the ith snail when they are sorted from smallest to largest.

RESULTS

There was a shift from no size hierarchy for *Potamopyrgus antipodarum* grown at no-competition to a strong size hierarchy for *P. antipodarum* grown with-competition (Fig. 3). The no-competition G was 0.251 and the mean with-competition G was 0.461 (95% upper and lower CI = 0.458, 0.465; min. = 0.286; max. = 0.650), which was significantly greater than the no-competition G (one-tailed t test for differences in means; N = 1000; t = 135.36; $p < 0.000$). The distribution of growth rates of *P. antipodarum* at no-competition was normally distributed (N = 20; Shapiro-Wilk W = 0.967; p = 0.696) but those grown with-competition were right-skewed and non-normally distributed (N = 200; Shapiro-Wilk W = 0.852; $p < 0.000$) (Fig. 3). Individuals of *P. antipodarum* grown at no-competition (mean growth = 0.338 mm/month, std. dev. = 0.146) grew significantly more (non-parametric Kolmogorov-Smirnov t-test, $p < 0.005$) than those grown with-competition (median growth = 0.100 mm/month, 25% quartile = 0.050, 75% quartile = 0.200).

DISCUSSION

Intraspecific competition for limited food resources caused decreased growth of *Potamopyrgus antipodarum* reared in the laboratory. One snail in our 22 cm^2 tube translates to roughly 455 snails/ m^2 and 10-snails/tube translates

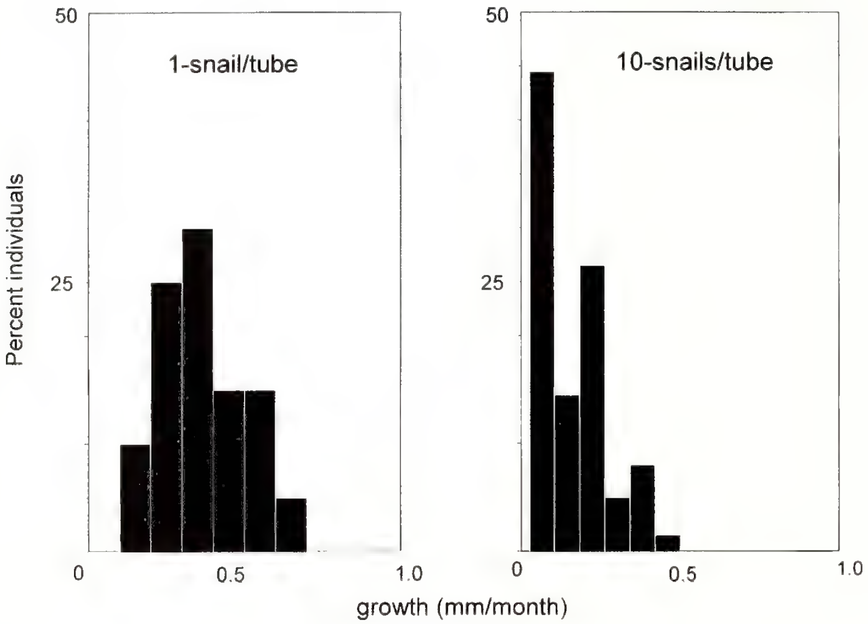

Figure 3. Growth (mm/month) of individuals of *Potamopyrgus antipodarum* with no competition (1 snail/tube; N = 20) and with intraspecific competition (10 snails/tube; N = 200).

to roughly 4555 snails/m^2, fairly low compared to naturally-occurring densities of *P. antipodarum*, which are often between 20,000 and 40,000/m^2 and occasionally exceed 500,000/m^2 in rivers in the western USA. Obviously, other environmental and ecological factors affect populations of *P. antipodarum* in waters in the western USA. It is likely, however, that intraspecific competition occurs in waters infested with *P. antipodarum*, particularly in late autumn and early winter when primary production is reduced and populations of *P. antipodarum* are large. Intraspecific competition may partially explain why many researchers report marked decreases in *P. antipodarum* densities and a scarcity of smaller individuals in winter (Dianne Shinn, pers. comm., David Richards, pers. comm., Billie Kerans and Chelsea Cada, pers. comm., and Daniel Gustafson, pers. comm.). We have shown that in a freshwater spring with fairly constant year-round temperatures (approx. 14°C) and flow rates that densities of *P. antipodarum* also decrease in autumn and winter, which indicates that temperature and flow are not entirely responsible (Richards *et al.* 2001).

In this experiment, asymmetric competition resulted in a well-defined size hierarchy. These results suggest that size hierarchies in populations of *Potamopyrgus antipodarum* in the western USA may not be entirely based on age class. Because asymmetric competition affects smaller individuals more than larger individuals, it might also result in reproductive hierarchies. Previously we found that in several rivers in the western USA, larger individuals of *P. antipodarum* produce more embryos; snails <3.00 mm did not reproduce at all (Richards *et al.* 2000). Reproductive hierarchies could result in self-thinning populations and/or dominant and suppressed size classes (Ford 1975, Ford and Diggle 1981). We do not know if the effects of inequality in individual reproductive output are greater than the effects of their inequality in size. Even though populations of *P. antipodarum* in the western USA are clonal, reproductive hierarchies could increase the proportion of genes represented by the larger, more fecund individuals in future generations (Heywood 1986, Damgaard and Weiner 2000).

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Behavior, morphology, and the coexistence of two pulmonate snails with molluscivorous fish: A comparative approach*

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Abstract: We conducted field, mesocosm, and laboratory studies to compare the behavioral and morphological defenses of two pulmonate snails, *Physa acuta* and *Stagnicola elodes*, against predation by pumpkinseed sunfish, *Lepomis gibbosus*. Field surveys showed that *P. acuta* occurred in most ponds and lakes in the study region, including those with and without fish. In contrast, *S. elodes* had a restricted habitat distribution, as it was found only in small ponds without fish. Behavioral plasticity of these two species was evaluated by monitoring their habitat use in mesocosms with and without caged pumpkinseed sunfish. Pumpkinseed sunfish induced a strong habitat shift by *P. acuta*, but *S. elodes* did not alter habitat use in the presence of caged pumpkinseeds. *Stagnicola elodes* has a stronger shell and a larger adult body size than does *P. acuta*. Handling time experiments showed that pumpkinseed sunfish had more difficulty consuming *S. elodes* than *P. acuta*. Despite the endowment of *S. elodes* with a relatively large, strong shell, it suffered higher mortality than did *P. acuta* in predation trials with pumpkinseed sunfish. However, the advantage of *P. acuta* disappeared when covered substrates were removed from the pools, showing that *P. acuta* rely on habitat shifts to minimize encounters with fish predators. Taken together, these results suggest that the mechanisms allowing *P. acuta* but not *S. elodes* to coexist with fish predators are largely behavioral rather than morphological.

Key words: anti-predator behavior, chemical cues, *Physa*, predation, *Stagnicola*

Understanding the mechanisms that underlie shifts in species composition along environmental gradients remains a central challenge of community ecology. The principal gradient in lentic freshwater systems is the environmental continuum from small, temporary ponds lacking fish to deep, permanent lakes containing fish. Freshwater snails, like most aquatic taxa, have characteristic species assemblages over different portions of this gradient (Pip 1986, Jokinen 1987, Dillon 2000). Although the mechanisms responsible for shifts in snail assemblages across this gradient are not well understood, work on other aquatic taxa has highlighted the role of predation in producing predictable patterns of species replacement along the size gradient of ponds (Wellborn *et al.* 1996, Skelly 1997, McPeek 1998). Very simply, smaller ponds tend to be temporary and lack a well developed predator assemblage. Traits that confer protection against the predators of larger ponds and lakes often incur costs that put the bearer of those traits at a disadvantage in small ponds lacking predators or containing different sorts of predators (e. g., Wissinger *et al.* 1999, Wellborn 2002). Although a large number of studies have produced shopping lists of traits that protect prey against predation, these studies typically focus on single traits in isolation, and they often fail to link traits to patterns of co-occurrence with predators in the field (Relyea 2004). Thus, while we know what sorts of traits may be used to deal with predators, we have a poor understanding of which sorts of traits are actually most important in allowing prey to coexist with predators and thus drive the process of species replacement.

Among the most important predators of pulmonate snails in lakes and ponds are molluscivorous fish (Brönmark *et al.* 1992, Martin *et al.* 1992, Brönmark 1994). Molluscivorous fish are most numerous in deeper, permanent ponds and lakes and are usually absent from shallow temporary ponds (Lodge *et al.* 1987). Pulmonate snails have a variety of adaptations that facilitate coexistence with fish predators. Some taxa possess relatively large, thick shells. These taxa are less preferred by fish than are thin-shelled taxa (Stein *et al.* 1984, Osenberg and Mittelbach 1989, French and Morgan 1995). Other taxa employ inducible anti-predator defenses. For example, field studies show pulmonates use safe microhabitats (covered substrates and shallow marginal areas) to a greater degree in lakes and ponds containing fish than in fishless ponds (Turner 1996, Bernot and Turner 2001) and that habitat use is a phenotypically plastic trait. Pulmonates use chemical cues to detect predators (Snyder 1967, Alexander and Covich 1991, Covich *et al.* 1994) and their be-

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havioral responses depend on the type of predator and their spatial and temporal proximity to the predator (Turner *et al.* 1999, McCarthy and Fisher 2000, Turner *et al.* 2000, Bernot and Turner 2001, Turner and Montgomery 2003). Pulmonates also respond to predators by altering their reproductive effort (Crowl and Covich 1990, Chase 1999), morphology (DeWitt 1998), and growth rates (Turner 1997, 2003).

In this paper we compare the behavioral and morphological defenses of two species of pulmonate snails, *Stagnicola elodes* (Say 1821), synonymous with *Stagnicola palustris* (Müller 1774), and *Physa acuta* (Draparnaud 1805), synonymous with *Physa integra* (Haldeman, 1841) and *Physa heterostropha* (Say, 1817), (Dillon *et al.* 2002). We present survey data showing that *S. elodes* is confined to small ponds that lack fish. *P. acuta* is also found in these same fishless ponds, but it occurs in ponds with fish as well. We hypothesize that *P. acuta*, but not *S. elodes*, possess morphological or behavioral adaptations that allow coexistence with fish predators. By comparing the antipredator adaptations of the two species, we hope to gain some insight into the way in which traits of individuals drive patterns of species replacement along the size gradient of ponds.

METHODS

Mollusc surveys

We present data extracted from an ongoing survey of snail assemblages of northwest Pennsylvania. We chose to focus our study on *Physa acuta* and *Stagnicola elodes* because each often dominates the snail assemblage of ponds in which they occur; more than 90% of the local lakes and ponds contain either *P. acuta* or *S. elodes*. Between 1997 and 2002 we sampled snails from 56 lakes and ponds in northwest Pennsylvania, encompassing a wide range of surface areas, depths, and hydroperiods. Sampling was generally conducted in May or early June. A D-frame sweep net was used to collect snails, and each pond was represented by at least 20 sweeps. The presence or absence of fish in a pond was ascertained through seining, dipnetting, minnow traps, or a combination thereof.

Behavioral plasticity

We assessed the relative behavioral responses of *Physa acuta* and *Stagnicola elodes* to predation risk by comparing their habitat use in a mesocosm experiment with and without caged pumpkinseed sunfish (*Lepomis gibbosus*). The pumpkinseed sunfish is a specialized molluscivore (Wainwright 1996, Huckins 1997), can have substantial effects on snail populations (Brönmark *et al.* 1992), and is ubiquitous in the smaller lakes and ponds of northeastern North America (Scott and Crossman 1973, Lee *et al.* 1980). Sixteen polyethylene pools (525 l, 1.4 m^2 surface area) were placed outdoors at the Pymatuning Laboratory of Ecology in northwest Pennsylvania and were filled with water from Pymatuning Reservoir. A square piece of corrugated vinyl (60 cm × 60 cm), raised off the bottom by legs that were 4 cm tall, was added to each pool to serve as habitat structure. Eight randomly selected pools were each stocked with 10 adult *P. acuta*; the other eight were stocked with 10 adult *S. elodes*. Both species were collected from an abandoned segment of the Erie Extension Canal near Linesville, Pennsylvania. The sampled reach lacked fish.

The species identity treatment was crossfactored with a predation risk treatment. "Fish present" pools received one pumpkinseed sunfish, confined to a cylindrical mesh cage (25 cm diameter × 75 cm length, 1-mm mesh). The mesh cage prevented pumpkinseeds from feeding on experimental snails, but allowed chemical odors emanating from the fish and their prey to disperse through the pool. Each day, caged pumpkinseeds were fed four *Physa acuta* or *Stagnicola elodes*, depending on the species treatment to which they were assigned to. "Fish absent" pools received empty cages.

Censuses of habitat use were conducted daily for seven days. We recorded the number of snails occupying near-surface habitats (within 2.5 cm of the water's surface, or above the water's surface), the number under the covered substrate, and the number remaining in other portions of the pool. Because earlier studies with *Physa acuta* show that they respond to fish predators both by moving under cover and by crawling to the water's surface (Turner 1997), we defined refuge use as the summed proportion of snails using these two habitats. The independent and interactive effects of predation risk and species identity on refuge use were analyzed with a 2 × 2 ANOVA. Preliminary analysis with repeated-measurements ANOVA showed no day-of-experiment effects or interactions of day-of-experiment effects with predator or species effects, so the analyses presented here were conducted on pool means (averaged over the seven day experiment).

Shell strength

The vulnerability of snails to pumpkinseed sunfish is largely a function of shell strength and body size (Osenberg and Mittelbach 1989). We compared the vulnerability of *Physa acuta* and *Stagnicola elodes* to pumpkinseed sunfish by measuring the force necessary to crush the shells of 45 randomly selected individuals of each species. Crush resistance was measured by placing a snail at the bottom of an upright cylinder (8 cm diameter) and inserting a piston in the cylinder and on top of the snail. The snail's shell was then subjected to a gradual increase in force by slowly filling the piston with sand until the shell failed. We positioned the snail such that the crushing force was oriented across the shortest shell dimension (dorsal to ventral), thereby best mimicking the feeding mode of molluscivorous fish (Wainwright 1996). The mass of the sand and piston was converted to Newtons, and using body mass as a covariate, we used ANCOVA to test whether size-adjusted shell strength differed between the two species. Studies show that shell strength, measured in this manner, is highly correlated with the time pumpkinseed sunfish spend handling prey (Osenberg and Mittelbach 1989, Huckins 1997).

The ability of gape-limited predators to ingest and crush snails depends on the snail's body size as well as shell strength (Osenberg and Mittelbach 1989, Nyström and Perez 1998), so we conducted foraging trials with pumpkinseeds feeding on snails and recorded handling times. Handling times are a good measure of the costs associated with foraging. Snails to be fed to pumpkinseeds were gathered from a local pond in late June; thus their size distributions reflected natural patterns. Eight pumpkinseeds, 120-130 mm standard length (SL), were fed *Physa acuta* and *Stagnicola elodes*. We recorded the time from ingestion of the snail to shell failure, which was marked by an audible sound. Handling times represented the average of approximately 100 individual trials for each species.

Predation trials

We evaluated the relative vulnerability of *Physa acuta* and *Stagnicola elodes* to pumpkinseed sunfish by conducting predation trials that encompassed both the encounter and handling phases of the predation process. To assess the potential role of behavioral defenses in minimizing encounter rates and thus ameliorating predation risks, we conducted trials with and without covered substrates present. The manipulation of habitat structure allowed us to assess the role of behavioral defenses and was an effective method of partitioning the relative roles of behavioral and morphological defenses. Trials were conducted in ten 525 l mesocosms placed outdoors at the Pymatuning Laboratory of Ecology. Each mesocosm was stocked with 10 *P. acuta* and 10 *S. elodes*. Half of the ten mesocosms contained a covered substrate in the form of a ceramic tile (20 cm square) elevated above the bottom on 2.5 cm tall legs and covering 3% of the pool bottom. One pumpkinseed (120-130 mm SL) was added to each mesocosm, where it was initially confined to a mesh-enclosed cage (30 × 30 × 30 cm). Caged pumpkinseeds were fed four snails daily for seven days (equally divided between *S. elodes* and *P. acuta*), thereby acclimating the fish to feeding on snails, and acclimating experimental snails to predation risk. After one week, the pumpkinseed was liberated and allowed to forage for four hours. Counts of snail survivorship were conducted after four hours of pumpkinseed foraging. Because both species were stocked into pools and pumpkinseeds were allowed to choose between them, their probabilities of survivorship were not independent of each other. Therefore, we analyzed survivorship data by first calculating the mortality rate of *S. elodes* relative to *P. acuta* (number of *S. elodes* eaten/[number of *S. elodes* eaten + number of *P. acuta* eaten]). Because the two species were stocked at the same density, this was equivalent to Manly's index of electivity, which has a value of 0.5 when there is equal preference for the two species (Manly *et al.* 1972, Chesson 1978). Values greater than 0.5 indicated a preference for *S. elodes* and values less than 0.5 indicated that *S. elodes* were avoided. We then used one-way ANOVA to test for the effect of habitat structure on pumpkinseed electivity.

RESULTS

Mollusc surveys

Field surveys show that *Physa acuta* occupies a broad range of habitats, whereas the distribution of *Stagnicola elodes* is quite restricted. Of 56 ponds and lakes surveyed to date, *P. acuta* was found in 48, but *S. elodes* occurred in just 5. *Physa acuta* was found both with and without fish, whereas *S. elodes* was only found in ponds generally lacking fish (repeated surveys of these ponds have yielded the occasional mudminnow, *Umbra lima*). Each of the five fishless ponds with *S. elodes* become dry by late summer in most years, but are shaded enough by trees that their soils retain some moisture (A. Turner, pers. obs.). Four of the five temporary ponds with *S. elodes* also contained *P. acuta*, the fifth contained its congener *Physa gyrina* (Lea 1838)—we distinguished *P. gyrina* from *P. acuta* by examining penile morphology. Both *P. acuta* and *S. elodes* could become quite abundant and dominate the snail assemblage of a pond. Sweep net samples taken in early spring, and thus comprised only of overwintering adults, showed that the density of *P. acuta* could exceed 10 snails per sweep. *S. elodes* densities could reach two snails per sweep (approximately 0.25 m^2 sampled per sweep).

Behavioral plasticity

Pumpkinseeds had a strong effect on habitat use of snails, but these effects were species specific (Fig. 1). Individuals of *Physa integra* increased their use of covered substrates from 41% to 92% when confronted with caged pumpkinseeds. In the absence of pumpkinseeds, covered habitat use by *Stagnicola elodes* was 42%, but increased to just 47% in the presence of pumpkinseeds, illustrating the differing behavioral responses of the two species to predation risk (species × predation risk interaction: $F_{1,12} = 24.3$, $P < 0.001$).

Shell strength

Body size, measured as dry mass of soft body parts, had a significant effect on shell strength (Table 1); the slope of

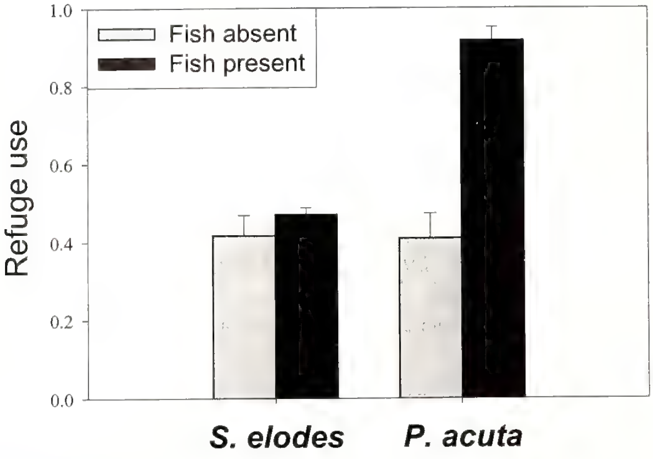

Figure 1. Refuge use by *Physa acuta* and *Stagnicola elodes* in the presence and absence of caged pumpkinseed sunfish. Refuge use is the summed proportion of snails using covered substrates and near-surface habitats and is averaged over seven days. Vertical bar represents one standard error, n = 4 mesocosms per treatment combination.

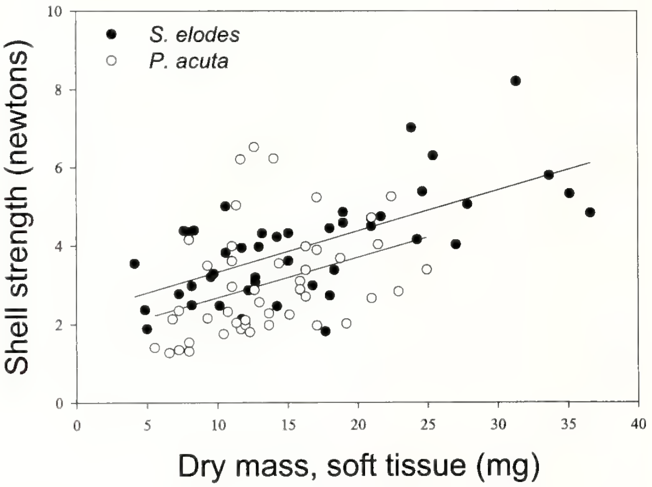

Figure 2. Force required to crush the shells of *Stagnicola elodes* and *Physa acuta* as a function of snail body size. Body size is dry mass of soft body parts (excluding shell material). See Table 1 for analysis of size and species effects.

the relationship was remarkably similar for the two species (homogeneity of slopes test, $P > 0.20$; Fig. 2). However, species identity did have a significant effect on size-adjusted shell strength (Table 1). Shells of *Physa acuta*, adjusted for size, failed at an average of 3.18 ± 0.16 (± SE) N, whereas *Stagnicola elodes* shells of the same size failed at 3.84 ± 0.17 N, an increase of 21%.

The trials to measure handling time confirmed that pumpkinseeds have more difficulty feeding on *Stagnicola elodes* than on *Physa acuta*. Handling time for *P. acuta* averaged 4.97 s, and handling time for pumpkinseeds feeding on *S. elodes* averaged 9.14 s (species effect in one-way ANOVA: $P < 0.01$). This difference may have been due in part to differences in the body sizes of the two species: shell height of *P. acuta* averaged 9.80 mm, whereas the shell height of *S. elodes* collected from the same pond averaged 19.3 mm.

Predation trials

In the absence of habitat structure, *Physa acuta* and *Stagnicola elodes* survived predation by pumpkinseeds at a similar rate (Fig. 3). Manly's index of electivity for pumpkinseeds foraging on *S. elodes* was 0.47 ± 0.04 (± SE), not significantly different from the equal preference value of 0.50. In pools containing habitat structure, however, survivorship of *P. acuta* was elevated more than 3-fold over pools without structure, whereas *S. elodes* survivorship was virtually unchanged (Fig. 3). Thus, adding habitat structure resulted in *S. elodes* suffering much higher mortality than *P. acuta*. Manly's index of electivity for pumpkinseeds feeding

Table 1. ANCOVA testing the effects of body size and species identity on shell strength. Model $R^2 = 0.36$

Source of variation	SS	df	MS	F	P
Body size	44.69	1	44.69	34.2	<0.00001
Species identity	9.68	1	9.68	7.4	0.0078
Error	117.59	90	1.31		

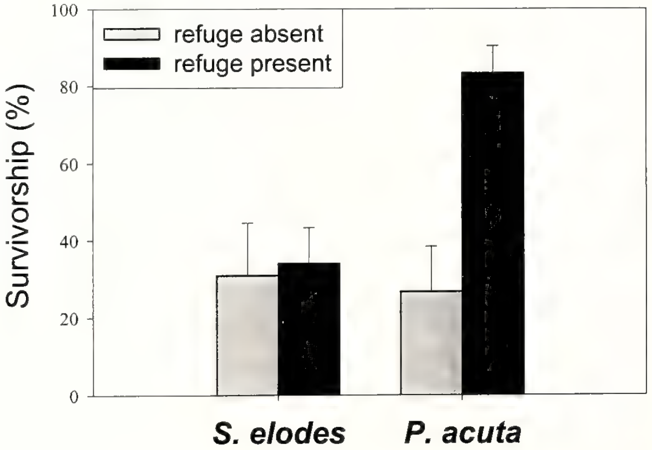

Figure 3. Survivorship of *Stagnicla elodes* and *Physa acuta* in experimental mesocosms when fed upon by pumpkinseed sunfish in the absence and presence of a covered substrate. Vertical bar represents one standard error, n = 8 mesocosms per treatment combination.

on *S. elodes* was 0.83 ± 0.07, showing strong preference (habitat structure effect on preference: $F_{1,9} = 16.3$, $P < 0.01$).

DISCUSSION

A number of malacologial studies have sought to link the degree of shell development with the ability to coexist with shell-crushing predators (Vermeij and Covich 1978, Palmer 1979, West *et al.* 1991). However, a naive malacologist approaching the freshwater habitat gradient and armed only with a knowledge of relative shell strength and predator distributions would fail to correctly predict the habitat distributions of *Stagnicola elodes* and *Physa acuta*. *Stagnicola elodes* has a relatively robust shell but occurs only in environments largely lacking in shell-crushing predators, whereas *P. acuta*, with its small thin shell, is most dominant in environments with abundant shell-crushing predators. Only knowledge of the relative behavioral flexibility of these two species allows an accurate prediction of their habitat distributions.

In other aquatic taxa, the predictable pattern of species replacement along the pond-size continuum is driven in large part by shifts in the identity of the top predator and tradeoffs involving traits conferring safety against predators (Bendell 1986, Skelly 1997, McPeek 1998). Unfortunately, almost nothing is known about the potential role of such mechanisms in structuring assemblages of snails. Descriptive studies aimed at detecting patterns in the species composition of assemblages of freshwater snails and identifying structuring mechanisms have traditionally focused on the importance of abiotic factors (Økland 1983, Brönmark 1985, Pip 1986, Jokinen 1987). On the other hand, a number of experimental field studies have tested the effects of predators on populations of snails (e.g., Brönmark *et al.* 1992, Martin *et al.* 1992, Osenberg *et al.* 1992, Lodge *et al.* 1994), but these studies have focused on regulations of populations within lakes, not patterns of replacement among lakes.

These results are consistent with the idea that fish prevent *Stagnicola elodes* from occupying a broader range of habitats, but other mechanisms may also be at work. *Stagnicola elodes* may be resource limited in permanent ponds (e.g., Eisenberg 1970) or interspecific competition may limit their distribution. Brown (1982) examined the competitive ability of *S. elodes* in relation to *Physa gyrina*, and concluded that *S. elodes* was the better competitor for food resources. In addition, Brown and DeVries (1985) found that *S. elodes* grew faster in a pond with fish than in a pond without fish. Thus, there is no evidence in this particular case that resources or competition limits the distribution of *S. elodes*.

Although this study is the first to compare carefully the traits of these two taxa, our results are consistent with earlier work. Brown (1982) also observed that *Stagnicola elodes* was restricted to temporary, partially wooded ponds. Brown and DeVries (1985) experimentally demonstrated that the central mudminnow *Umbra limi* depresses populations of *S. elodes* and concluded that fish predation limits their habitat distribution. Interestingly, Brown and DeVries (1985) found that *U. limi* was capable of feeding only on the very smallest *S. elodes* (<3 mm shell length), so any population regulation of *S. elodes* by mudminnows must occur during a short but deadly juvenile bottleneck. Snyder (1967) included *S. elodes* and *Physa acuta* in his comparative study of predator avoidance and found that although *S. elodes* showed some response to intraspecific extract, their reaction was considerably weaker than that of *P. acuta* (Snyder 1967: tables 8-9). *Stagnicola elodes* in Snyder's study showed no response to pumpkinseed sunfish, whereas *P. acuta* responded strongly to the same cues.

The fact that our study involved just two taxa limits the generality of our conclusions. There is a clear need for more comprehensive comparative studies of this sort, involving more taxa and perhaps controlling for phylogenetic effects. The available data suggest, however, that behavioral defenses may ultimately prove to play a preeminent role in driving patterns of species replacement. For example, in eastern North America, *Helisoma trivolvis* (Say 1817) is also broadly distributed across habitat types, and it moves to safe habitats in the presence of predators (Alexander and Covich 1991, Turner 2003). Two studies have shown that the degree of behavioral flexibility is correlated with shell strength such that thin-shelled taxa respond more strongly to predators than do thick-shelled taxa (Snyder 1967, Rundle and Brönmark 2001). In our study region (northwest Pennsylvania) there occur several species of snails with quite stout shells, including *Bithynia tentaculata* (Linnaeus, 1758), *Campeloma decisum* (Say, 1816), and *Elimia livescens* (Menke, 1830). These taxa are quite restricted in their habitat distributions and are not necessarily found in habitats with abundant shell crushing predators (A. Turner, pers. obs.). Snyder (1967) performed assays of predator avoidance for each of these three thick-shelled taxa and found no evidence for behavioral plasticity. On the other hand, taxa with broad habitat distributions tend to be small, relatively thin-shelled taxa (e.g., Planorbidae, Physidae, Lymaeaidae). In fact, we observed that *P. acuta* and *H. trivolvis* are most dominant relative to other snail taxa in lakes or ponds with abundant predators (A. Turner, pers. obs.). Thus, it appears that it is the behaviorally "reactive" species that are most successful in the presence of predators.

A number of studies involving molluscs and their predators have successfully linked interspecific comparisons of shell strength and vulnerability to predators (Stein *et al.* 1984, Osenberg and Mittelbach 1989, West *et al.* 1991, French and Morgan 1995, Brown 1998). These are short-term foraging trials conducted in aquaria. Perhaps not surprisingly, these studies show that prey with poorly developed morphological defenses suffer higher mortality from predators than do well defended prey taxa. However, these studies have quite abbreviated spatial and temporal scales and limited opportunities for prey to use refugia, and thus do not allow for the expression of behavioral or life-historical defenses. In addition, few studies have presented survey data in conjunction with measurements of morphology and vulnerability to predators. It is possible that the traits allowing coexistence with predators are primarily behavioral, life-historical, or physiological, and not morphological, in which case the community ecology of a system is not predictable from measurements of morphology.

Investigators have long been puzzled by the general lack of shell development in freshwater snails. With the exception of snail faunas endemic to ancient lakes and rivers, freshwater snails tend to have much thinner shells than do marine snails and tend to be smaller. This pattern has been attributed to differences in predation intensity: perhaps freshwater environments lack specialized shell-crushing molluscivores and contain fewer predators than do marine environments (Vermeij and Covich 1978). However, studies of aquatic food webs show that several widespread and abundant predatory taxa are in fact shell-crushing molluscivores (reviews in Lodge *et al.* 1987, Dillon 2000, Brown 2001), and a number of experiments now demonstrate that molluscivorous fish, crayfish, and other shell-crushing predators can have large effects on the population size and assemblage composition of snails (reviews in Dillon 2000, Brown 2001). In view of the growing literature showing rampant phenotypic plasticity in the traits of pulmonate snails, the emerging picture is that freshwater snails have quite elaborate defenses, but that these defenses are (1) largely inducible, as opposed to constitutive and (2) behavioral, as opposed to morphological.

We suggest that it is not differences in mean predation risk but instead differences in the spatial and temporal heterogeneity of predation pressure in freshwater that may in part account for the reliance of pulmonate snails on inducible defenses. Phenotypic plasticity allows simultaneous adaptation and cost minimization across broad environmental gradients. In addition, behavioral traits are readily reversible, whereas induced shifts in shell characters are not. One would expect then to see the largest deployment of phenotypically plastic traits to prevail in environments in which predation pressure shows the most spatial and temporal heterogeneity. A full comparative analysis of the concordance of shell development, phenotypic plasticity, patterns of coexistence with predators, and gene flow will offer valuable insights.

Finally, we echo Dillon (2000) and point out that community-level studies of freshwater snails lag behind those of other aquatic taxa. This is not because the system is intractable. Indeed, given the relative ease with which one can measure their morphological, behavioral, life-historical, and physiological traits, estimate their abundance in the field, and conduct appropriately scaled mesocosm and field experiments, freshwater snails clearly present an outstanding opportunity to link mechanistically the traits of individuals to community-level patterns. We look forward with optimism to future studies of snail community ecology.

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Effects of pair-type and isolation time on mating interactions of a freshwater snail, *Physa gyrina* (Say, 1821)*

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Abstract: Behavioral tendencies during mating interactions can have important effects on mating patterns. Factors hypothesized to influence mating behavior include the degree of genetic similarity between mates and sexual motivation. This study tested for effects of these factors on mating interactions of the aquatic snail *Physa gyrina*. Three types of pairs were created from two populations of snails (i.e., two types of intrapopulation pairs and one type of interpopulation pair) and individuals experienced one of three isolation treatments. I recorded behavioral dynamics during mating interactions, including: escalations of mating behaviors, error frequencies, rejection behaviors, and mating frequency. Mating interactions were influenced by both the pairing treatments and isolation treatments. Additionally, there were significant interaction effects between the experimental factors on the behavioral dynamics between individuals during mating interactions. Interpopulation pairs had greater avoidance rates than intrapopulation pairs and differed from control pairs in a variety of ways. Altering the amount of isolation time individuals experienced also significantly affected the behavioral dynamics. Isolation resulted in decreased avoidance responses, with concomitant increases in the escalations of interactions and numbers of copulations. Matings occurred sooner after longer isolation periods, but also experienced higher error frequencies, e.g. misalignment during copulation attempts. These results indicate that the type of potential mate and the context in which interactions occur are important considerations when interpreting observations of mating interactions.

Key words: *Physa*, mating behavior, mate assessment, temporal patterns, body size

A major focus in behavioral ecology is the study of mate choice. Most animals do not mate indiscriminately, but prefer some potential mates to others (Halliday 1983, Andersson 1994, Johnstone 1997). Tremendous effort has been made to increase our understanding of mating processes and how mate-choice decisions translate into fitness benefits (Bateson, 1983, Andersson, 1994). Hermaphroditic snails provide an excellent system for examining questions related to mating behaviors during interactions (Leonard 1991, DeWitt 1996, Wethington and Dillon 1996, Baur and Baur 1997, Wethington *et al.* 1999) and their fitness consequences (Jarne and Delay 1990, Chen 1993, Monsutti and Perrin 1999), as well as the effects of contextual influences on both behavior and fitness (Chen 1993, Schrag *et al.* 1994a, 1994b, De Boer *et al.* 1997, Locher and Baur 2002).

One factor that could potentially affect mate choice is genetic similarity (Bateson 1983). Genetic similarity between mates varies widely (Waser 1993). Individuals may encounter close relatives as potential mates (Barnard and Fitzsimons 1988, 1989, Waldman *et al.* 1992) with genotypes similar to their own (e.g. siblings), or the genotypes of potential mates can differ considerably from that of the individual. Outbreeding may occur between unrelated individuals within a population or between members of different populations via immigration and emigration processes. Pair-types (e.g. inbred versus outbred matings), in conjunction with migration events, might strongly impact the genetic structure of a population. However, surprisingly few studies have examined how mate-choice decisions are mediated by recognition of kinship or how inbreeding or outbreeding affect the fitness consequences of those decisions (Sherman *et al.* 1997).

The contexts in which mating interactions occur also influence mating behavior and mate choice (Halliday 1983, Westneat *et al.* 2000) and can have important fitness implications (Chen 1993, Keller *et al.* 1994, Pray *et al.* 1994, Armbruster *et al.* 1997, Baur and Baur 1997). Recent sexual experience may influence mating interactions. Some studies show that mate-choice preferences are clear when sexual motivation is low, but with high sexual drive (e.g. virgins or individuals with low sperm stores) choosiness regarding mates may be low, and consequently, mate-choice preferences are not exhibited (Halliday 1983). That is, sexually motivated individuals may be less choosy about the quality of potential mates than recently mated individuals (Tomiyama 1996). For example, Wethington *et al.* (1999) found no behavioral isolation between individuals from different species, but they used virgin snails in their study. Discrimination or choosiness might not be expected, even between members of different species, when sexual motivation is high. However, it is also possible that changes in sexual motivation levels, and thus shifts in behavioral tendencies, might occur without significant depletion of sperm stores.

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Aquatic snails are an ideal model system for studying mating behavior and reproductive fitness. *Physa* is a genus of aquatic snails common throughout North America (Burch 1989). Individuals of *Physa* are often easy to collect in the field and maintain in the laboratory. Because individuals of *Physa* are simultaneous hermaphrodites, every mature individual, including itself, is a potential mate. Species capable of both cross- and self-fertilization are excellent systems for investigating factors that maintain outcrossing within populations (Schrag *et al.* 1994a). Additionally, physid snails can store sperm from matings for long periods (>60 days: Wethington and Dillon 1991). Individuals of *Physa* mate readily, produce large numbers of offspring, and their mating behavior is easily observed (DeWitt 1991).

The primary goal of this study was to examine whether two factors, pair-type and isolation time, influenced mating interactions. I also considered potential mechanisms that could have led to observed differences between treatments: changes in encounter probabilities, mating behaviors, or rejection rates. A second goal of this study was to determine whether there was evidence indicating significant interactions between these factors during mating encounters. These questions were addressed by observing mating interactions of aquatic snails in the laboratory.

METHODS

This experiment examined the effects of pair-type and isolation time (3 × 3 factorial design) on the mating interactions of mature individuals of *Physa gyrina* (Say, 1821). Snails were collected from two local populations: Buck Run Creek (BRC) in Woodford County, KY, and from a small drainage ditch running parallel to train tracks (TT) near the University of Kentucky campus. Snails were held in 38-l aquaria, fed boiled lettuce *ad libitum*, and treated for 5 days with an antibiotic (Maracyn®) to minimize disease. Individuals from BRC were generally larger than those from TT (mean shell length ± SE: BRC = 10.92 ± 0.16 mm, TT = 10.01 ± 0.08 mm, *t*-test: $t_{178} = 5.20$, $P = 0.001$) but the size ranges did overlap.

Ninety snails from each population were randomly assigned to one of three pairing treatments: BRC pairs, TT pairs, or interpopulation pairs (BRC × TT). I marked individuals' shells with a paint pen to indicate the population of origin. I then formed snail pairs by partnering individuals that were similar in size (shell length); partners were closely matched for size and the mean proportional size differences of paired snails did not differ between pairing treatments (percent shell-length differences of partners ± SE: BRC = 3.0 ± 0.4%, TT = 4.0 ± 0.5%, BRC × TT = 4.0 ± 0.5%, ANOVA: $F_{2,87} = 1.96$, $P = 0.15$). Because BRC snails were generally larger than TT snails, there were significant differences in mean sizes (= average shell length of pair) of paired snails in the three pairing treatments (ANOVA: $F_{2,87} = 13.14$, $P = 0.001$), a Tukey post-hoc test indicated that BRC pairs were larger than TT and interpopulation pairs.

Pairs were then assigned to one of three time treatments: no isolation time (0 day), one day of isolation (1 day), and three days of isolation (3 day). Individuals from pairs that were assigned to the 1- and 3-day isolation treatments were isolated in separate containers for the appropriate length of time. Snails assigned to the 0-day isolation treatment were used immediately after pair formation. That is, individuals were taken from their holding tanks, marked, assigned a partner, and then observed in the trials without any isolation time.

During the experiment, paired snails (n=10 pairs per pairing*time treatment) were placed in Petri dishes containing 50 ml of clean water and observed for 60 minutes. Behavioral dynamics and interaction outcomes were recorded. DeWitt (1991, 1996) described the behavioral dynamics that occur during mating interactions: snails physically contact each other, one snail will then assume the male gender role by mounting the shell of the second individual, the "male" then crawls in circles on the shell of the "female" until in the proper alignment for attempting a copulation, the "male" will then evert the intromittent organ (the preputium), and a copulation occurs when the preputium contacts and transfers sperm to the gonopore of the "female" (sperm can be observed in the preputium). While these are essentially "male" behaviors, DeWitt also described a variety of rejection or resistance behaviors that are gender-neutral (e.g. avoidance response—individual avoids further interaction following a contact by abruptly changing crawling direction or swinging its shell away from the other snail) or conducted by snails occupying the "female" gender role (e.g. lateral shaking of the shell or abruptly drawing shell down to substrate).

I quantified the behavioral dynamics by calculating mean conditional frequencies of escalation behaviors for each pair during the observation period as follows: number of contacts per hour, number of mountings per contact, number of positioning behaviors per mounting, number of preputium eversions per mounting, number of "male errors" per mounting (i.e. male everts preputium when not properly aligned along aperture of female's shell), number of copulations per preputium eversion. I also quantified resistance and rejection responses by calculating mean conditional frequencies: number of avoidance responses per number of contacts, number of "female" resistance behaviors per mounting, and number of "male rejections" per mounting (i.e. male dismounts female without attempting to copulate and without resistance behaviors from the female).

I compared mating behaviors between pair-type and isolation-time treatments with two-way ANCOVAs. Failure-time analyses (Fox, 1993) compared the mean elapsed times until successful copulations occurred in each treatment. Statistical tests were run with SYSTAT 8.0. The ANCOVA models used "mean size" and proportional "size difference" (= percent size difference in shell length) of paired individuals as covariates. The two covariates were used to account for any behavioral differences between pairs ("mean size") or individuals within pairs ("size difference") due to differences in body size (DeWitt 1996, DeWitt *et al.* 1999). I used Pearson correlation matrices to show the nature of the effect when the covariates were significant. When the ANCOVAs indicated significant treatment effects, I used Tukey post-hoc tests to compare responses between factor-levels within treatments. If the ANCOVAs found significant treatment interaction effects, I used Fisher's Least Significant Difference (LSD) post-hoc tests and then corrected for the number of multiple comparisons of interest (18 comparisons of interest: I compared pairing treatments within time treatments and effects of time within pairing treatments, $\alpha \approx 0.003$). Failure-time analyses, which compare the rates at which interactions occur, may give an indication of mating preferences that are not reflected in behavioral transition rates described above. That is, a treatment factor may influence the speed at which behaviors occur and interactions escalate without affecting the mean conditional frequencies.

RESULTS

Both the pair-type and isolation-time treatments had significant effects on behavior. Encounter rates differed between pair-types and were influenced by the isolation treatments (Table 1, Fig. 1A). The TT pairs had fewer contacts than the other pair-types. Encounter rates also decreased with increasing isolation time. There was no significant in-

Table 1. Results of ANCOVA's that tested for effects of pair-type and isolation time on various aspects of mating interactions in *Physa gyrina.* The 0-day isolation treatments were dropped from the analyses as indicated below (*) due to low preputium eversion rates.

			Covariates				Treatment effects					
			Mean size (df = 1)		Size difference (df = 1)		Pair type (df = 2)		Isolation time (df = 2)		Pair*Time (df = 4)	
	R-Squared	Error df	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Encounter Rate												
Contacts/hour	0.371	79	13.73	**<0.001**	1.53	0.22	5.27	**<0.01**	8.79	**<0.001**	0.78	0.54
Behavioral Transition Rates												
Mounting/contact	0.350	79	7.11	**<0.01**	0.12	0.73	2.36	0.101	12.46	**<0.001**	1.02	0.40
Positioning/mounting	0.330	77	7.07	**<0.05**	1.62	0.21	3.58	**<0.05**	7.21	**<0.001**	2.71	**<0.05**
Preputium eversion/ mounting	0.298	77	4.37	**<0.05**	2.20	0.14	4.06	**<0.05**	5.81	**<0.005**	2.06	0.09
"Errors"/preputium*	0.382	35	0.29	0.60	6.26	**<0.05**	2.14	0.13	4.72	**<0.05**	0.56	0.58
Copulations/preputium*	0.197	35	1.20	0.28	1.20	0.28	1.44	0.25	1.97	0.17	1.51	0.24
Rejection Transition Rates												
Avoidance/contact	0.288	79	0.86	0.36	0.21	0.65	6.49	**<0.005**	7.77	**<0.001**	0.97	0.43
Resistance/mounting	0.242	77	5.06	**<0.05**	0.01	0.93	2.15	0.12	3.16	**<0.05**	2.72	**<0.05**
"Male rejection"/ mounting	0.433	77	13.24	**<0.001**	2.97	0.09	4.79	**<0.05**	12.19	**<0.001**	3.12	**<0.05**
Interaction Outcome												
Copulation number	0.272	79	6.76	**<0.05**	0.01	0.96	3.44	**<0.05**	7.31	**<0.001**	0.97	0.43
Copulation duration	0.157	36	0.07	0.79	0.50	0.48	0.17	0.84	0.20	0.82	0.95	0.45

* 0-day isolation treatment excluded from analysis

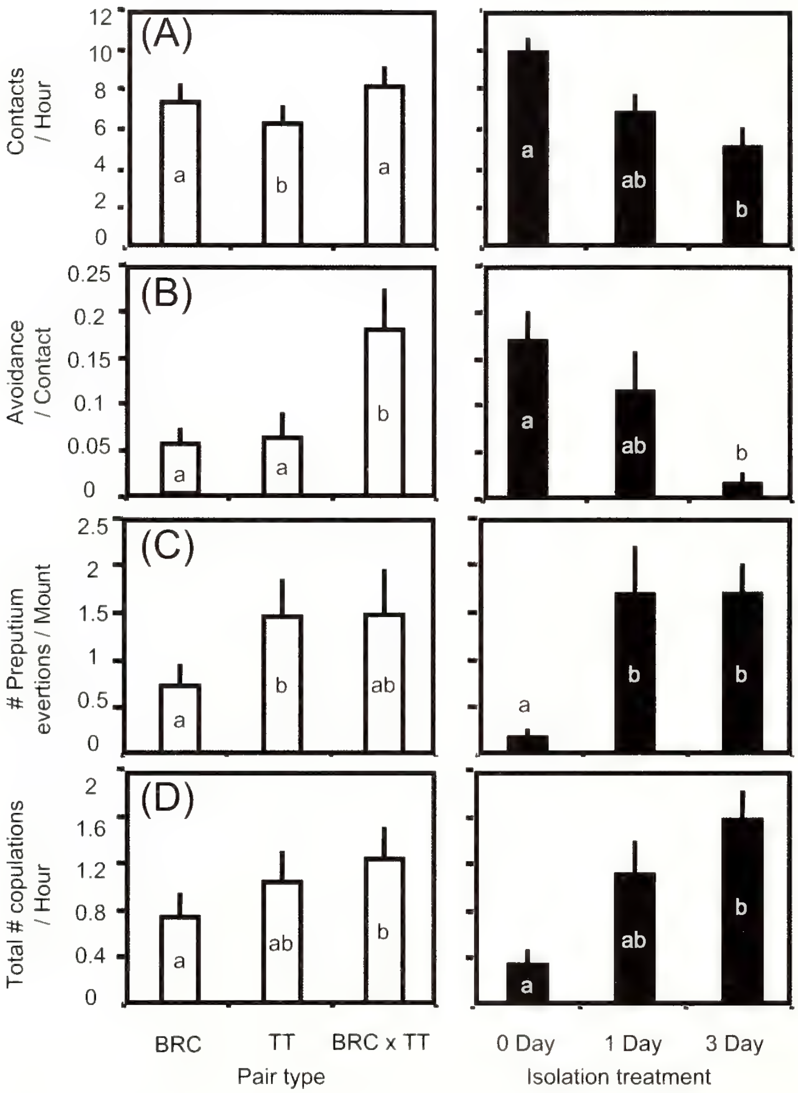

Figure 1. Mean number (± SE) of (A) physical contacts per hour, (B) avoidance responses per contact, (C) preputium eversions per mounting, and (D) copulations per hour between paired individuals of *Physa gyrina* in each pair-type (BRC: Buck Run Creek population, TT: train tracks population) and within each isolation time treatment. Bars sharing the same letter within a panel are not statistically different.

teraction between pair types and isolation times. However, the mean size of the individuals in the pair had a significant effect on the number of encounters (Table 1), there was a negative correlation between size and encounter rate (Table 2).

Following an encounter, the probability (mean ± SE) of one snail mounting its partner did not differ between the pair types: BRC pairs = 0.49 ± 0.05, TT pairs = 0.57 ± 0.09, BRC × TT pairs = 0.53 ± 0.08. In contrast, avoidance rates differed significantly between the pair types (Table 1). Snails in the interpopulation pairs showed greater avoidance responses than did the intrapopulation pairs (Fig. 1B). Isolation time also significantly influenced avoidance and mounting behaviors (Table 1). Mounting behaviors increased (0-day = 0.28 ± 0.03, 1-day = 0.54 ± 0.07, 3-day = 0.76 ± 0.09) and avoidance responses decreased (Fig. 1B) in frequency with longer isolation times. Mean size of the paired individuals was positively correlated with mounting responses (Table 2). That is, snails in larger pairs were more likely to mount.

Male positioning behaviors were significantly influenced by pair-type, isolation time, and the interaction between these factors (Table 1). Although the interpopulation pairs did not differ significantly from the intrapopulation pairs, the TT pairs had a significantly higher positioning frequency than did the BRC pairs in the 1-day treatment (Fig. 2A). The positioning frequencies of the snails in the three pairing treatments did not differ significantly in the 3-day treatment. Again, the mean size of the paired snails was positively correlated with the frequency of positioning behaviors (Table 2).

"Male rejections" (i.e. dismounting "female" without attempting to copulate and without female resistance) were also significantly influenced by pair-type, isolation time, and the interaction between these factors (Table 1, Fig. 2B). "Males" in all pairing treatments had similar rejection patterns, such that rejection frequencies decreased if snails were isolated ($\alpha = 0.003$ for multiple comparisons, BRC: 0-day > 3-day, $P = 0.001$, TT: 0-day > 1-day, $P = 0.003$, BRC × TT: 0-day > 3-day, $P = 0.001$). There were no significant differences between pair-types in the 0-day treatment. Although the interpopulation pairs did not differ significantly from the intrapopulation pairs, the TT pairs had a significantly lower "male rejection" frequency than did the BRC pairs in the 1-day treatment. The "male rejection" frequencies of the pairing treatments were not significantly different in the 3-day treatment. Furthermore, fewer male rejections were observed in pairs with larger individuals (Table 2).

The propensity of males to attempt copulations by everting the preputium differed between pair-type and isolation treatments (Table 1). Snails in the BRC pairs attempted fewer copulations per mounting than those in the TT pairs. Additionally, snails that had not been isolated (0-day) had significantly fewer preputium eversions than did individuals that had been isolated (Fig. 1C). Mean size was

Table 2. Summary of results from Pearson correlation matrices that examined the effects of covariates ("mean size" and proportional "size difference") on behaviors during mating interactions. Positive values indicate a positive relationship between the covariate and the response variable, while negative values indicate a negative relationship (ns = non-significant).

		Pearson correlation coefficients	
Response variable	# obs.	Mean size	Size difference
Contacts/Hour	90	−0.321	ns
Mounting/Contact	90	0.245	ns
Positioning/Mounting	88	0.179	ns
Preputium eversion/Mounting	88	0.131	ns
"Errors"/Preputium	43	ns	−0.414
Copulations/Preputium	43	ns	ns
Avoidance/Contact	90	ns	ns
Resistance/Mounting	88	0.149	ns
"Male rejection"/Mounting	88	−0.259	ns
Copulation number	90	0.18	ns
Copulation duration	47	ns	ns

positively correlated with the frequency of preputium eversions (Table 2). Interestingly, increasing "error" rates (i.e. misalignment during preputium eversions) were observed the longer snails were isolated (Table 1: 0-day = 0.06 ± 0.06 [note that 0-day treatments were omitted from the analyses due to the extremely low frequency of preputium eversions and are only included here for comparison], 1-day = 0.11 ± 0.05, 3-day = 0.24 ± 0.05). "Error" rates were also influenced by the proportional size differences between individuals within pairs, such that similarly sized snails had higher error rates than pairs with greater size discrepancies (Table 2). The rates of successfully copulating, given an attempt (as above, 0-day treatments were omitted), were not influenced by either pair type or isolation time (Table 1).

"Female resistance" behaviors (lateral shell shaking or quickly drawing shell to substrate following a mounting by a "male") were significantly influenced by isolation time and the interaction between pair-type and isolation time (Table 1). Females in the TT and interpopulation pairs had similar resistance patterns. The frequency of resistance behaviors by females in the BRC pairs was significantly lower than those of females in the other pairing treatments (Fig. 2C). Additionally, the mean size of the paired individuals was positively correlated with female resistance (Table 2).

The total number of matings that occurred during the 1-hour observation period was influenced by both the pair-type and the isolation time (Table 1). Interpopulation pairs had a greater number of copulations than did BRC pairs and the number of copulations increased with longer isolation times (Fig. 1D). Copulation number was positively correlated with the mean size of the pairs (Table 2). Failure-time analyses indicated that matings also occurred sooner with increasing isolation time (Fig. 3). However, duration of copulation was not influenced by any of the treatments (Table 1).

DISCUSSION

The results of this study indicate that the context in which mating interactions occur has important implications for behavioral dynamics and interaction outcome. The experimental treatments significantly affected mating interactions of *Physa gyrina*. The types of individuals present in the pairs influenced behavioral transitions during interactions. Additionally, short-term periods of isolation had strong effects on mating interactions. There were also significant interaction effects between the pair-type and isolation-time treatments. Furthermore, both body size and the magnitude of differences in body sizes of pair mates influenced mating interactions.

Pair-type treatments were constructed to examine whether the presumed genetic similarities of pair-mates would influence mating interactions. Typically, individuals will interact with others from their own population, but there may be opportunities to interact with immigrants (or emigrate themselves). I assumed that individuals within the intrapopulation pairs (i.e. BRC pairs and TT pairs) would be more similar genetically than those in interpopulation pairs (Dillon and Wethington 1995). However, it is important to note that the snails may not be assessing the degree of genetic similarity of potential mates, but rather some other aspect of the phenotype (e.g. environmentally influenced traits).

As a whole, the results suggest that potential mates are being assessed and treated differently. Interpopulation pairs had the greatest mean number of copulations (significantly greater than BRC pairs, Fig. 1). Snails acting in the male gender role in interpopulation pairs tended to have higher frequencies of positioning behaviors and fewer "male rejection" behaviors (note that the lack of significant differences between interpopulation pairs and intrapopulation pairs for these behaviors is likely due to the low α-value [$\alpha = 0.003$] resulting from the corrections for multiple comparisons, e.g. test comparing positioning behaviors of BRC pairs versus BRC × TT pairs was not significant in 1-day treatments: $P = 0.008$). This differs from previous experiments that have demonstrated that snails prefer individuals from their own populations as mates (Baur and Baur 1992, Rupp and Woolhouse 1999). In support of these studies, I also found that interpopulation pairs had the highest frequencies of avoidance behavior and tended to have higher frequencies of "fe-

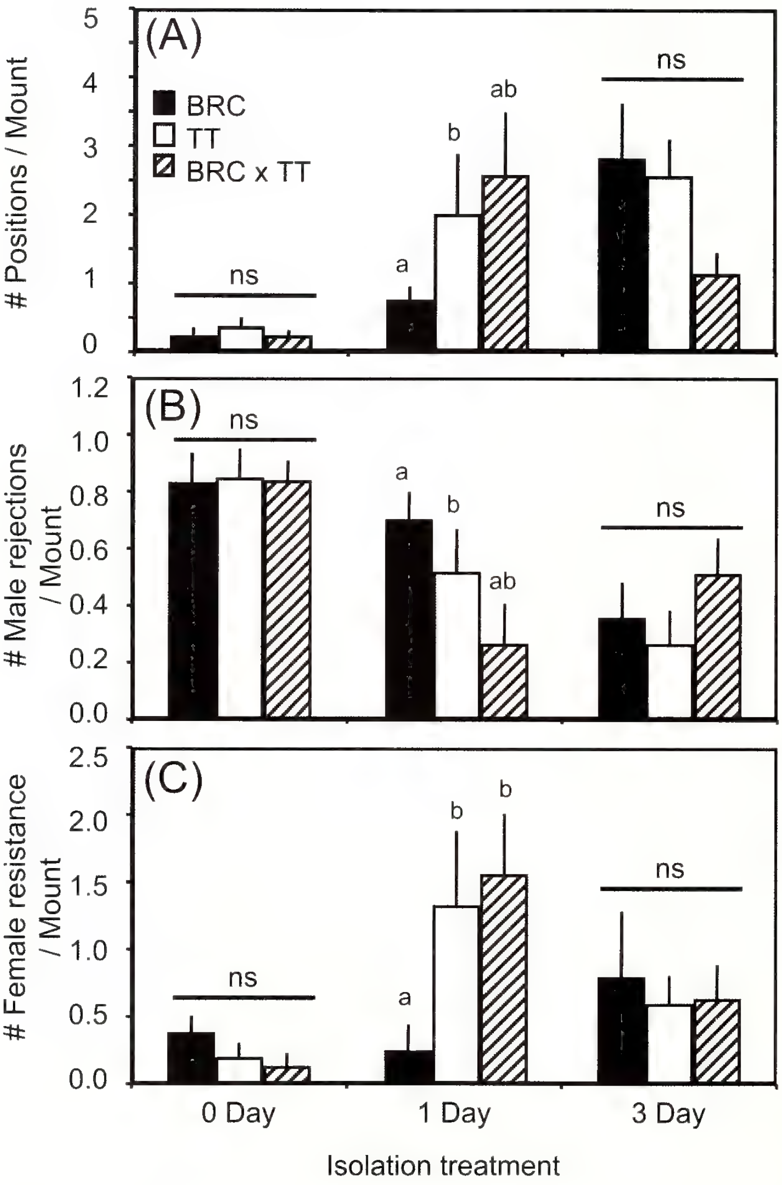

Figure 2. Behavioral responses of individuals of *Physa gyrina* observed during mating interactions: (A) mean number (± SE) of positioning behaviors per mounting, (B) the proportion (± SE) of individuals acting in male gender role that rejected their partner without attempting to copulate, and (C) mean number (± SE) of resistance behaviors per mounting by individuals acting in the female role. See text for descriptions of "male rejection" and "female resistance." Bars sharing the same letter within a panel are not statistically different (ns = non-significant differences within isolation treatment, BRC: Buck Run Creek population, TT: train tracks population).

male resistance" (significantly greater than BRC pairs in 1-day isolation treatments, Fig. 2).

Numerous recent studies have theoretically or empirically addressed some of the interesting complexities of mating interactions between hermaphroditic individuals, especially with regards to gender allocation, choice, or conflict (e.g. Leonard 1991, DeWitt 1996, Wethington and Dillon 1996, Crowley *et al.* 1998, Angeloni *et al.* 2002, Locher and Baur 2002). The behavioral tendencies discussed above suggest that individuals may have asymmetrical gender-based mating preferences. In this experiment snails seemed to prefer interpopulation partners while acting in the male gender role, but also resisted interpopulation partners while occupying the female gender role. The avoidance of interpopulation partners during gender-neutral situations (following a contact) may be a mechanism to avoid becoming the "female" during an interaction.

While it is beyond the scope of this study to discuss in any detail the possible mechanisms underlying population-level differences, there are several lines of evidence suggesting that the populations may have subtle differences in their mating behaviors. First, the population pairs differed significantly in contact rates (BRC > TT) and the frequency of preputium eversion (TT > BRC). Secondly, the patterns of response to isolation treatments differed between the population pairs for a number of the response variables. For instance, after one day of isolation the TT pairs had higher positioning frequencies than BRC pairs, and TT "females" also had higher frequencies of resistance than did BRC snails. However, neither of these behaviors differed significantly between intrapopulation pairs in the 0- and 3-day isolation treatments. Interestingly, this suggests that individuals from separate populations may behave similarly under one set of circumstances but exhibit dissimilar behavioral patterns in a slightly different context.

Isolation periods were intended to manipulate the sexual motivation of the snails (Wethington and Dillon 1996, De Boer *et al.* 1997) and might simulate low population density situations in the field. As expected, there were strong temporal effects on behavior, isolation reduced avoidance, increased frequencies of mounting and preputium eversion, and increased the number of copulations. Matings occurred sooner the longer snails were isolated (Fig. 3), but surprisingly there was also a greater error rate during preputium eversion. These behavioral patterns are consistent with the expectations for increased sexual motivation.

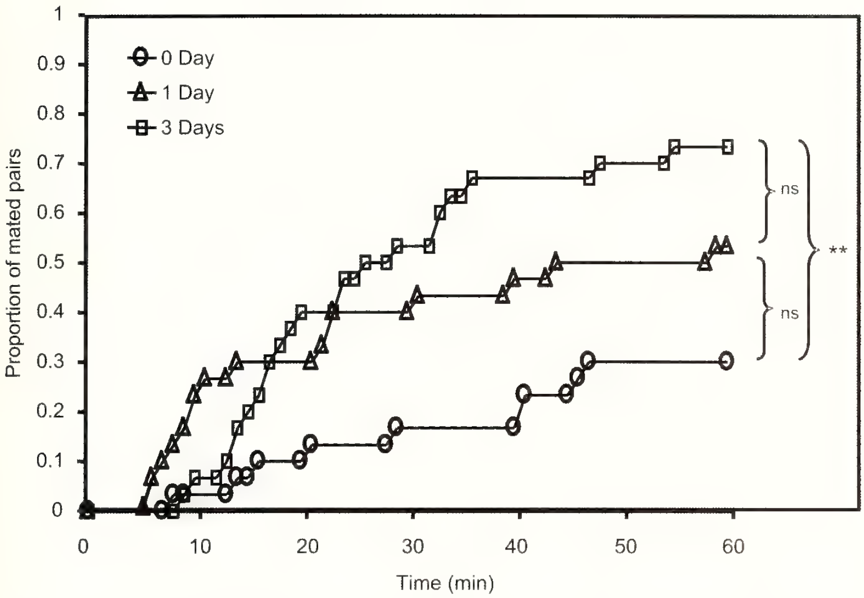

Figure 3. Mating distribution functions of time depicting the proportions of pairs of *Physa gyrina* that had mated within each isolation-time treatment. (ns = non-significant, ** = $P < 0.01$).

Isolation time also had unexpected effects on behavior. First, the numbers of contacts decreased with increasing isolation time. The simplest explanation for this effect is that the durations of interactions increased with isolation time so that there was less time during the observation period for subsequent interactions. Second, the behavioral patterns, while often not statistically significant, suggest that isolation time might differentially influence motivational levels of the gender roles. For instance, the data indicate that "male" escalation behaviors typically increased after one day of isolation, which suggests elevated motivational levels. But snails acting as females generally had greater frequencies of resistance behaviors in 1-day treatments, which implies that "female" motivational levels had not been influenced to the same degree as "male" sexual motivation. A possible explanation for the observed effects is that there is a trade-off between mating preferences and sexual motivation, such that, choosiness decreases as motivation increases (Halliday 1983, Tomiyama 1996). Consequently, the sexual motivation of a snail (as a "male") increased after one day of isolation but it was still relatively choosy (when acting as "female") about the quality of potential mates. Interestingly, resistance behaviors were not as frequent in the 3-day treatments suggesting that "female" motivation had increased even though it is unlikely that the snails were experiencing sperm depletion. Again, this suggests gender-differences within individuals, and the asynchrony of increased motivational levels may be caused by distinct mechanisms. For example, "male" motivation can be influenced by the build-up of sperm (De Boer *et al.* 1997), whereas "female" motivation might be influenced by the quantity or maturity of eggs.

Individual body sizes and the size differences between mates were important factors in mating interactions. Theory predicts that both the size of an individual and the size of its mate should influence gender strategies during mating interactions of simultaneous hermaphrodites (Angeloni *et al.* 2002). Both mating behaviors during interactions and copulation frequency were positively correlated with the mean body sizes of paired individuals in this study. This suggests mating effort increases with body size. Surprisingly, the proportional size differences of mates were negatively correlated with errors during preputium eversion. That is, closely size-matched pairs had higher error rates than pairs in which individuals were more disparate in size.

It is also interesting to note that as size increased the frequencies of "male" rejection decreased while those of "female" resistance increased. These phenomena could be interpreted in a number of ways. For example, the findings might imply that as an individual's body size increases, it is less likely to reject potential mates when occupying the male role and more likely to resist as a female. However there is an alternative explanation for these findings since paired individuals were similar in size. Individuals acting in the male role may be less likely to reject large females and females may be more likely to reject large males. For instance, DeWitt (1996; also see Angeloni *et al.* 2002) found that smaller snails typically occupied the male gender role while larger snails acted as females. DeWitt also observed greater rejection rates when males were larger than females.

This study demonstrates that both the type of individual encountered and isolation time can influence the behavioral dynamics and outcomes of mating interactions. It also illustrates some of the gender complexities of hermaphroditic systems. There are likely to be many factors, such as migration rates and population densities, influencing mating interactions in wild populations. Furthermore, there may be unexpected interactions between these factors. Therefore, the context-dependent nature of the interactions suggests that we must be careful about how experimental findings are related both to other laboratory studies and to field observations.

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Comparative conservation ecology of pleurocerid and pulmonate gastropods of the United States*

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Abstract: To understand better the conservation status of freshwater pleurocerid gastropods in the southeastern United States, we contrasted their distribution and biology with pulmonate snails, a group far less imperiled. With 157 taxa in North America, the Pleuroceridae have a similar species richness to all pulmonate families combined (153 taxa). The Pleuroceridae has more recently extinct species (38) and 66% of the remaining taxa are considered imperiled (G Rank ≤ 2). Pleurocerid species richness is greatest in the Alabama and Tennessee River basins in the southeastern United States, with only 25 species outside the southeastern United States. We argue that the endemic nature of their distributions, limited dispersal, and poor abilities to colonize heighten chances of extinction for pleurocerids in comparison to pulmonates. We also outline our approach to conservation, which involves delineating appropriate habitat parameters for successful re-introductions using artificial propagation. We call for a national conservation strategy for pleurocerids, emphasizing threats to lotic pleurocerids and how management agencies can surmount them, similar to the strategy already developed for unionid bivalves.

Key words: Re-introduction, Artificial Propagation

The pleurocerids (Gastropoda: Cerithioidea) are an imperiled group of freshwater snails limited to streams and rivers in the southeastern United States. Their biology contrasts with that of a sister taxon, pulmonate snails, which have comparatively healthy populations and are found throughout the United States. Critical differences in pleurocerid ecology make these snails more susceptible to extinction, with recent extinctions of 38 species. Conservation of remaining populations of pleurocerids will involve assessment of their habitat requirements, artificial propagation, and reintroduction of populations to suitable habitats.

METHODS

Taxonomic nomenclature used in this manuscript follows Turgeon *et al.* (1998). We based our assessment of the original distribution and diversity of gastropods in part on the monograph by Burch (1989), augmented with recent field data for many species in the Tennessee and Mobile River basins. Burch's monograph includes collection data from malacologists in the nineteenth and twentieth centuries and was compiled from data originally assembled by Calvin Goodrich from 1922-1944. Burch reviewed all species' names for synonymy and grouped taxa that were obvious examples of excessive "splitting." We therefore consider Burch's monograph to be the best available document to assess the original distributions of gastropods before extensive anthropogenic impacts.

Data on the number of extinctions in each group are from Neves *et al.* (1997) and the conservation status of remaining populations are primarily drawn from databases initially prepared for The Nature Conservancy and now maintained by NatureServe (2004). For designating conservation status of extant species, we use the G ranking system developed by Heritage/Conservation Data Center Network and The Nature Conservancy (Stern 2002). The higher the G rank a species receives, the greater the number of known populations. We consider that any G rank of two or less constitutes an imperiled species. A brief explanation of the conservation rank categories are:

GX—Presumed Extinct: Species is believed to be extinct throughout its historic range.
G1—Critically Imperiled: Species with 5 or fewer occurrences, and few remaining individuals.
G2—Imperiled: Species occurrences are rare (6-20) throughout the known range.
G3—Vulnerable: Species typically has 21-100 occurrences within known range.

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* From the symposium "The Biology and Conservation of Freshwater Gastropods" presented at the annual meeting of the American Malacological Society, held 3-7 August 2002 in Charleston, South Carolina, USA.

G4—Apparently Secure: Species is uncommon but not rare, with more than 100 occurrences in a wide geographic range.

G5—Secure: Species is common with wide geographic range and abundant occurrences within range.

We consider species with a GX rank to also be imperiled. In the absence of complete field data on North American freshwater gastropods, current information on species distribution is limited and many areas remain unsampled. Future sampling may locate species now considered extinct, especially those that are extremely rare or have small geographic ranges. This exact scenario has occurred twice in the last 12 years, with the rediscovery of *Tulotoma magnifica* (Conrad, 1834) (Hershler *et al.* 1990) and *Leptoxis downiei* (Lea, 1843) (P. Johnson, pers. comm.), both of which were thought extinct in the Coosa River basin.

The NatureServe (2004) data bases themselves were originally based on Burch's monograph, but distributional data and G rankings for southeastern "prosobranch" species in particular have been evaluated and modified at 4 meetings of field investigators sponsored by The Nature Conservancy and the Alabama Division of Wildlife and Freshwater Fisheries (Mirarchi *et al.* 2004). These databases undoubtedly contain limitations, but again are probably the best indicator we have of the current distributions and diversity of gastropods. For pleurocerids, distributional data from the Tennessee and Mobile River basins are the most exhaustive and current, and information about species endemic to the Gulf Coastal drainages are the probably the most unreliable, since these populations have received less sampling effort.

RESULTS

Conservation status of freshwater gastropods

Extinction rates in molluscs are higher than more-publicized rates in vertebrate groups like fish, birds, reptiles, or mammals. There have been 51 recent gastropod, and 34 recent unionid mussel extinctions, in comparison to 30 recent extinctions in the vertebrate groups combined. Of the remaining molluscan populations, a much higher percentage are imperiled than in other freshwater groups such as fish, amphibians and crayfish. Sixty percent of extant gastropod, and 49 percent of extant unionid mussel species are at risk, in comparison to values of 21-33% for the other aquatic taxa.

Two families of North American freshwater gastropods, the Hydrobiidae and the Pleuroceridae, appear to be the most at risk (Table 1). Hydrobiids are currently considered to be the most diverse group of freshwater gastropods in the United States, based on recent species descriptions from springs and headwaters in the southeastern and western United States. The endemic nature of their distributions explains why a high proportion of these populations are at risk, especially if a species has been collected at only a few sites. Pleurocerids are the second most diverse group of freshwater snails in the United States and the southeast is the center of their diversity. However, the family Pleuroceridae also has the greatest number of recently extinct species for any group of North American snails (Table 1). Of the eight genera comprising the Pleuroceridae, one is extinct and two others have lost half of their species (Table 2). In comparison, most pulmonate families have few if any species that have gone extinct and extant populations are usually large, as is also the case for the families Valvatidae and Viviparidae.

Table 1. Comparative statistics on diversity, extinctions, and conservation status of North American freshwater "prosobranchs" and pulmonates. Data adapted and updated from NatureServe databases and Turgeon *et al.* (1998). Imperiled species are those with rankings of G2 or less.

Family	Number of species	Number of extinctions	Percent imperiled species
"Prosobranchia:"			
Hydrobiidae	239	8	85
Neritinidae	1	0	0
Pleuroceridae	157	38	66
Pomatiopsidae	6	0	67
Viviparidae	25	0	28
Pulmonata:			
Ancylidae	13	0	31
Lymnaeidae	56	0	23
Physidae	40	0	23
Planorbidae	44	5	5
Valvatidae	10	0	40
Total	590	51	60

Currently, 21 species of freshwater gastropods are federally listed as threatened or endangered (Table 3) and most have had recovery plans formulated. Alabama has the greatest number (10) of federally listed species. However, 95 taxa (51% of extant species) in Alabama were recognized as being in need of conservation at a recent Alabama Division of Wildlife and Freshwater Fishes (ADWFF) state wildlife meeting (Mirarchi *et al.* 2004). In general, endangered species are clustered either in the rivers in the southeastern United States (both pleurocerids and hydrobiids) or in isolated springs in the western states (hydrobiids).

Based on distributional data from Burch's (1989) monograph, pleurocerids are obviously most diverse in river systems in the southeastern United States (Fig. 1). Alabama is home to 101 species, Tennessee 41, Georgia 24, and Ken-

Table 2. The conservation status of the North American Pleuroceridae by genus. Data were updated from NatureServe (2004) databases and a recent assessment by the Alabama Division of Wildlife and Freshwater Fishes (Mirarchi *et al.* 2004).

Genus	Species	G1	G2	GX	Percent imperiled
Athearnia Morrison, 1971	2	1	0	1	100
Elimia H. and A. Adams, 1854	85	20	15	15	59
Gyrotoma Shuttleworth, 1845	6	0	0	6	100
Io Lea, 1831	1	0	0	0	0
Juga H. and A. Adams, 1854	9	5	1	0	67
Leptoxis Rafinesque, 1819	24	5	3	12	83
Lithasia Haldeman, 1840	11	2	5	1	72
Pleurocera Rafinesque, 1818	19	4	8	0	63
Species total	157	37	32	35	
Pleuroceridae percent total		23.5	20.3	22.3	66.1

tucky 20. The Coosa River in northeastern Alabama originally had 45 species, followed in diversity by the Tennessee, Cahaba, Cumberland, and Ohio Rivers. The Coosa, Cahaba, and Tallapoosa Rivers are in the Alabama River drainage system, probably the world's most diverse system for freshwater gastropods.

In comparison, pulmonate species are much more widely dispersed (Fig. 2). Pulmonates are most diverse in the northern tier of states in the United States. Even the tenth most diverse assemblage, in Wisconsin, has over 30 species, and pulmonates are quite diverse and abundant in Canada as well (Clarke 1981).

Pleurocerids and pulmonates also differ greatly in the sizes of their geographic ranges (Fig. 3). Most pleurocerids are found in only one state. Pulmonates show a bimodal distribution, with some species restricted to only a few states and others being widespread. Most pulmonate species are found in at least three states. These data must be taken with the caveat that some "species" probably have restricted distributions simply because they are not true species but are eco-morphs described in a particular stream or pond based only on differences in environmentally plastic traits like shell structure. This may also explain the bimodal distribution pattern in pulmonates to some extent.

Table 3. Distribution of federally listed freshwater gastropods as of 2002.

State	Number of species listed	Families represented
Alabama	10	Hydrobiidae, Pleuroceridae, Viviparidae
Idaho	6	Hydrobiidae, Lymnaeidae, Physidae, Valvatidae
Missouri	1	Hydrobiidae
New Mexico	2	Hydrobiidae
Tennessee	2	Hydrobiidae, Pleuroceridae

In light of this distributional difference between the two groups, it is not surprising that G rankings also differ (Fig. 4). The modal G ranking for pleurocerids is G1 and the median is G2,compared to a mode and median of G5 for pulmonates. These data indicate clear differences in conservation status between the two groups of gastropods, with pleurocerid species much more at risk.

Distributional patterns within river systems have not been studied extensively in either group. However, one study suggests that the groups may differ in micro-habitat use as well. Brown *et al.* (1998) sampled gastropod assemblages from headwater sites in the Salt River system in central Kentucky to the confluence of the Salt River with the Ohio River. All sampling sites were grouped by river order (e.g., the number of tributaries flowing into them). For example, a first order stream is a headwater stream, which is often ephemeral in this system, drying late in the summer to form a series of non-connected pools. Pulmonates (*Physa* spp. and *Helisoma* spp.) were only found in low order systems (Fig. 5). *Elimia semicarinata* (Say, 1829) was widely distributed and occurred in all but the largest river reaches. The other two pleurocerid genera, *Pleurocera* Rafinesque, 1818 and *Lithasia* Haldeman, 1840, occurred only in the higher-order reaches.

DISCUSSION

Differences in "prosobranch" and pulmonate biology

Why do "prosobranch" and pulmonate gastropods differ so dramatically in conservation status? First, these two groups of gastropods have quite different evolutionary pathways. Freshwater "prosobranch" gastropods apparently evolved from species physiologically adapted to the lower osmotic concentrations in estuaries and then dispersed into freshwater environments (McMahon 1983, Brown 2001). This adaptive radiation occurred before continental drift had separated modern continents. In contrast, terrestrial pulmonates apparently evolved from species living in the high intertidal zone that adapted to semi-terrestrial environments. The evolution of a simple lung allowed pulmonates to become completely terrestrial. Pulmonates then secondarily invaded freshwater habitats.

As one might expect from these divergent evolutionary histories, the biologies of pulmonates and freshwater "pro-

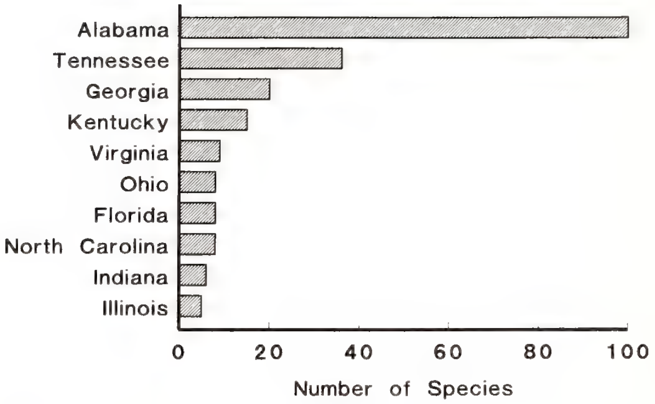

Figure 1. Number of pleurocerid taxa found in the ten states with the greatest biodiversity. Data compiled from Burch (1989).

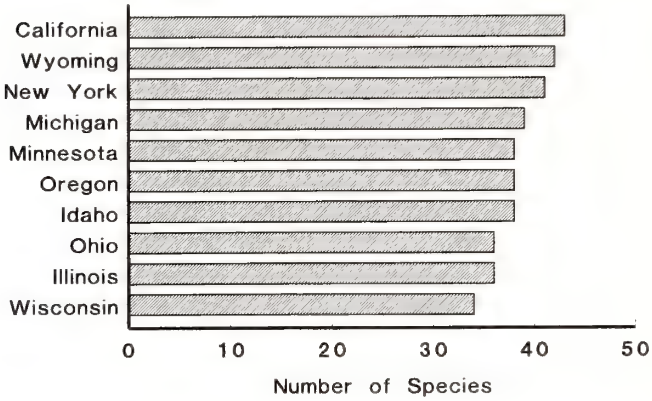

Figure 2. Number of pulmonate taxa found in the ten states with the greatest biodiversity. Data compiled from Burch (1989).

sobranchs" are quite different. Pulmonates are mostly annual species, with high fecundities and hermaphroditic reproduction (Brown 1983, Dillon 2000). Pleurocerids, on the other hand, are perennial, iteroparous, and dioecious (Huryn *et al.* 1994, Brown 2001). Its lung evidently allows a juvenile pulmonate to be passively and widely dispersed in the mud clinging to birds' feet (see references in Brown 2001). Freshwater prosobranchs, in contrast, have much slower and more restricted adult dispersal along river courses, perhaps explaining higher levels of genetic differentiation and endemism (Davis 1982). Another major difference involves the degree of risk of predation. Pleurocerids have thick shells and opercula that decrease their risk to invertebrate predators and fish (Lodge *et al.* 1987). Pulmonates have thin shells that are easily crushed by fish (Stein *et al.* 1984) or crayfish (Brown 1998) and the lack of an operculum increases predation risk to invertebrate predators as well (see references in Brown 2001).

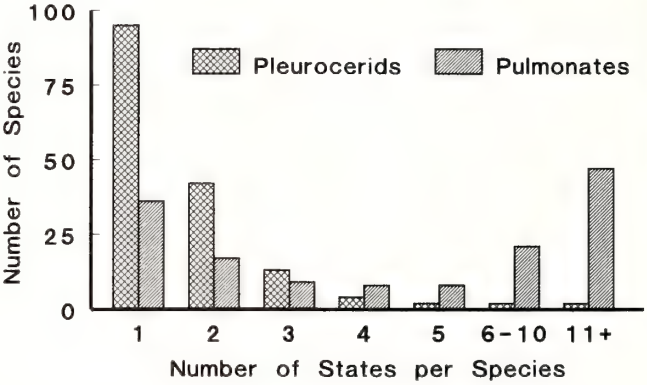

Figure 3. Distribution of species ranges for pleurocerid and pulmonate gastropods in the United States. Data plotted are the total number of states in which each species occurs. Data compiled from Burch (1989).

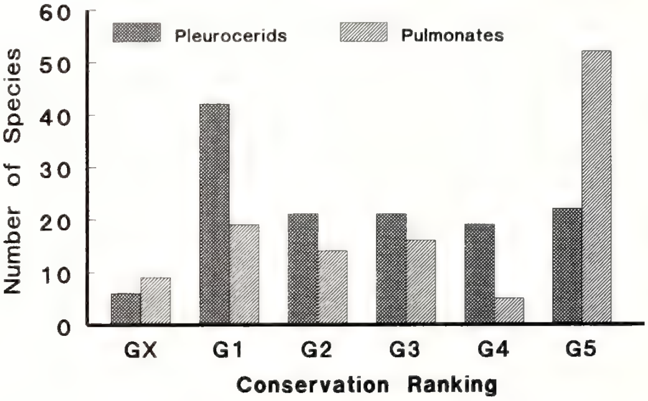

Figure 4. G rankings for extant populations of pleurocerid and pulmonate gastropods in the United States. GX stands for extinct. Rankings with higher numbers refer to species with greater numbers of known populations. Data compiled from NatureServe (2004).

Populations of both gastropod groups are food limited, based on a number of studies in which periphyton resources were experimentally manipulated. In pond-dwelling pulmonates and lotic "prosobranchs," either adult density or individual growth rates respond positively to food additions or density reductions (see references in Brown 2001). Some lotic pleurocerids (i.e., *Elimia* spp. and *Pleurocera* spp.) have lower adult abundances and smaller individual sizes in swift-flowing streams, evidently because higher flow rates dislodge larger snails or interfere with movement and feeding (Johnson and Brown 1997). In *Lithasia* spp., *Leptoxis* spp., and *Io fluvialis* (Say, 1825), in contrast, larger snails are common in areas with high current velocities (P. D. Johnson, pers. comm.).

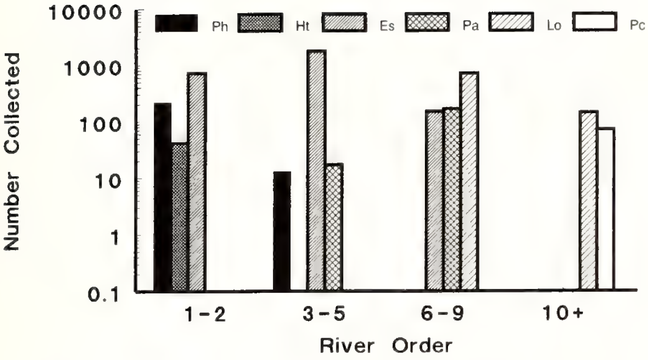

Figure 5. Distribution patterns of freshwater gastropods in the Salt River system in central Kentucky. Number of specimens collected for each species are arrayed against river order. Note that pulmonates (Ph = *Physa hendersoni* [Clench, 1925], Ht = *Helisoma trivolvis* [Say, 1817]) are common in headwaters, while pleurocerids (Es = *Elimia semicarinata*, Lo = *Leptoxis obovata* [Say, 1829], Pa = *Pleurocera acuta* Rafinesque, 1831, Pc = *Pleurocera canaliculatum* [Say, 1821]) are common in larger rivers. Data from Brown *et al.* (1998).

With their more "r-selected" life histories, higher dispersal rates, and hermaphroditic habits, pulmonates are ideally suited to colonize and survive in ephemeral habitats like temporary ponds, ditches, and other lentic environments (Brown *et al.* 1998). Pleurocerids and other prosobranchs are restricted to rivers and lakes because of lower dispersal rates or are more common in these permanent habitats because they are more resistant to predation (Brown *et al.* 1998).

Recovery efforts

A two-pronged approach to conservation of imperiled populations of gastropods is necessary. First, detailed information on distributions and habitat requirements is necessary. Such data can be used to assess whether sites within the species' historic range are still suitable for re-introduction of artificially propagated populations. Important micro-habitat characters include, but are not limited to, water depth and hardness, current velocity, substrate particle size, periphyton production, water quality, and habitat stability (Johnson and Brown 1997, Brown 2001).

Second, adequate propagation methods must be developed. Although translocations have been successful with *Io fluvialis* (Ahlstedt 1991), this option works only when the extant population is large. For many pleurocerids, especially in the Alabama River basin, sufficient individuals cannot be translocated without endangering the original population, and captive propagation is necessary. For each species, temperature and flow regimes must first be evaluated to determine the best combination to induce egg laying and juvenile growth. Propagation techniques for several genera of pleurocerids (*Elimia* H. and A. Adams, 1854, *Io* Lea, 1831, *Leptoxis* Rafinesque, 1819, and *Pleurocera*) are under development at the Tennessee Aquarium Research Institute in Cohutta, Georgia. In 2002, over 8000 juvenile *Leptoxis plicata* (Conrad, 1834), a federally listed species, were produced from 100 adults held for 3 months. In October 2002, 2734 lab-propagated *I. fluvialis* were released into the Tennessee River below Nickajack Reservoir, in Marion County, Tennessee.

In summary, pleurocerids are most at risk because of their limited dispersal powers. The limited movement by adults has undoubtedly played a role in geographically isolating populations, promoting speciation and resulting in the high diversity of the group. Pulmonates, by comparison, are fairly cosmopolitan, but less diverse. Their shorter life cycles (allowing rapid population growth), hermaphroditism, and high fecundity promote greater dispersal and successful colonization across river basins.

The more endemic nature of pleurocerids has thus targeted them for increased chances of extinction. But what are the ecological threats to persistence? They are probably much the same as those responsible for high extinction rates in unionids (Williams *et al.* 1993, Vaughn and Taylor 1999). Many pleurocerids are abundant in shallow riffles (shoals) in rivers, where conditions are quite favorable: warm, oxygenated water, large substrate particle sizes (providing refugia where snails can survive spates), sediment-free substrates, and maximal periphyton production. Impoundments change micro-habitats dramatically; the same sites often become hypoxic, with extremely cold water, high siltation rates, and loss of periphyton food. Large impoundments may also limit adult migration among the last remaining pristine stretches of rivers and thus increase fragmentation of populations.

We suggest that a national strategy for freshwater snail conservation is needed, similar to the one developed for unionid mussels (National Native Mussel Conservation Committee 1997). Information is needed on which species are most in peril and what plans resource managers should implement to assure survival of these species, including habitat restoration, conservation of existing populations, captive propagation, and re-introduction. The strategy should emphasize threats to extant populations and stress both public outreach and inter-agency cooperation.

ACKNOWLEDGEMENTS

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Reproductive isolation between *Physa acuta* and *Physa gyrina* in joint culture*

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Abstract: Recent laboratory tests of postzygotic reproductive isolation in physid snails, although providing fresh insight into the evolution of an important model organism, have focused on reproductively compatible populations of *Physa acuta*. Here we extend such studies to include a population of *Physa gyrina* known to be incompatible with *P. acuta*. Reared in pairs, the median age of first reproduction in a laboratory population of *P. acuta* originating from Charleston, South Carolina, USA was nine weeks. Over the next ten weeks of reproduction, the laboratory population of *P. acuta* posted a mean fecundity of 61.9 embryos per pair per week, with a mean F_1 viability of 63% and 100% F_1 fertility. Individual *P. acuta* reproduced by self-fertilization when reared with *P. gyrina* in no-choice mating experiments. Their median age at first reproduction was delayed to 10.5 weeks, their fecundity was 36.4 embryos per parent per week, and F_1 viability reduced to 26%. These figures were not significantly different from the reproductive success of individual *P. acuta* self-fertilizing in isolation (median 11 weeks at first reproduction, 37.4 embryos per parent per week, 37% hatchling viability, 88% F_1 fertility). Laboratory populations of *P. gyrina* originating from Hot Springs, Virginia, USA, were not as well adapted to our culture conditions as *P. acuta*. Pairs did not initiate egg laying until a median age of 11.5 weeks, after which their mean fecundity was only 21.2 per pair per week over ten weeks, with an F_1 viability of 33.5% and 100% F_1 fertility. When reared with *P. acuta* in joint culture, individual *P. gyrina* did not reproduce successfully. Thus the effects of joint culture with *P. gyrina* were negligible for *P. acuta* but ruinous for *P. gyrina* reared with *P. acuta*. These results have important implications for the interpretation of experiments involving postmating reproductive isolation with no-choice design.

Key words: *Physa*, Basommatophora, Pulmonata, mating, speciation

Freshwater pulmonates of the family Physidae may simultaneously be counted among America's best-known and least-known gastropods. Their adaptability to laboratory culture has led to great strides in our understanding of genetics (Dillon and Wethington 1992, 1994, Monsutti and Perrin 1999), morphology (DeWitt *et al.* 1999), life history (Rollo and Hawryluk 1988, Crowl and Covich 1990, McCollum *et al.* 1998), ecology (Brown *et al.* 1994, Turner *et al.* 2000, Bernot and Turner 2001), reproductive biology (Jarne *et al.* 2000, Wethington and Dillon 1993, 1997), and behavior (Covich *et al.* 1994, Wethington and Dillon 1996, DeWitt 1996, Turner *et al.* 1999, McCarthy and Fisher 2000). For a review see Dillon (2000). Yet their genetic diversity and phenotypic plasticity has resulted in confusion regarding the specific identity of even the most widespread American physid taxa.

Recently we have initiated a program of laboratory breeding experimentation designed to assess reproductive isolation among a variety of physid populations worldwide. We have established that two of the nominal species most common in North America, *Physa heterostropha* (Say, 1817) and *Physa integra* (Haldeman, 1841), are conspecific with European populations of *Physa acuta* (Draparnaud, 1805) (Dillon *et al.* 2002). Our no-choice mating experiments yielded no evidence of delay in maturity or reduction in fecundity, F_1 viability, or F_1 fertility among hybrids of six populations (two of each species) below incross controls. Experimental crosses such as these will, however, be more reliably interpreted given the benefit of a "negative control." Thus the purpose of the present experiment was to document the reproductive activity of pairs of physids known to be reproductively isolated when cultured in a no-choice design.

Physa acuta is a member of the subgenus *Costatella* Dall, 1870 (Burch and Tottenham 1980), characterized by a two-part penial sheath. *Physa gyrina* (Say, 1821), a member of the subgenus *Physa* (*s.s.*), has a three-part penial sheath. The animals are similar in their overall morphology; *P. gyrina* maturing at a slightly larger size and bearing a more rounded shell with more convex apical whorls. Individuals of the two species will copulate, although our preliminary observations have suggested that the only progeny born are the products of self-fertilization, rather than hybridization (Wethington *et al.* 2000).

The most likely outcome of jointly culturing a pair of non-hybridizing physids in a no-choice design would seem to be reproduction below that posted by control pairs of either species. Self-fertilization, which would be the only reproductive option expected in this experimental situation, is known to engender delayed age at first reproduction, reduced fecundity, and reduced hatchling viability in physids generally (Jarne *et al.* 1993, 2000, Wethington and Dillon 1997). Moreover, a pair of mismatched *Physa* Draparnaud, 1801 might be expected to compete with each other for food and other resources, and perhaps interfere with the self-fertilization process through false copulation, yielding a reduction in fecundity below even control individuals self-fertilizing in isolation.

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* From the symposium "The Biology and Conservation of Freshwater Gastropods" presented at the annual meeting of the American Malacological Society, held 3-7 August 2002 in Charleston, South Carolina, USA.

It is also possible, however, that self-fertilization in one or both individuals might be "socially facilitated" by a second snail present in joint culture, even if not conspecific. Vernon (1995) observed that, although self-fertilization reduces reproductive success in the planorbid *Biomphalaria glabrata* (Say, 1818), the reproductive output of snails reared in pairs but prevented from cross fertilizing by a nylon mesh barrier may approach that of outcrossing pairs. The ordinarily self-fertilizing terrestrial pulmonate *Balea perversa* (Linné, 1758) enjoys increased longevity and reproductive success when cultured with a partner, even though paired snails do not copulate (Baur and Baur 2000). Such social facilitation might also occur between snails as similar as *Physa acuta* and *Physa gyrina.*

Our investigation thus included four treatments: an experiment and three controls. The reproductive success of the *acuta* × *gyrina* experiment was compared to *acuta* × *acuta* controls, *gyrina* × *gyrina* controls, and a self-fertilizing control of *Physa acuta* reared in isolation. This design allowed us to identify social facilitation even in the reduced reproductive output expected from a pair of non-hybridizing species.

METHODS

Our population of *Physa acuta* was collected at Charles Towne Landing State Park (32°49′N, 79°59′W), west of the Ashley River within the city limits of Charleston, South Carolina. Animals from this population (previously identified as *Physa heterostropha* [Say, 1817]) have been the subject of many of our past studies on the genetics (Dillon and Wethington 1994, 1995) and reproductive biology (Wethington and Dillon 1991, 1993, 1997) of the genus *Physa*. Our population of *Physa gyrina* was collected in the town of Hot Springs, Virginia, approximately 100 meters downstream from the origin of naturally-heated waters inside "The Homestead" resort (38°36′N, 79°30′W). This is the type locality of *Physa aurea* (Lea, 1838), now recognized as a subspecies of *P. gyrina* (Burch and Tottenham 1980).

Our standard culture vessel was a transparent polyethylene 295.73 ml (10 US oz.) drinking cup, which we filled with approximately 210 ml of aerated, filtered pond water and covered with a 95 × 15 mm polystyrene Petri dish lid. The food was O. S. I. *Spirulina* Aquarium Flake Food, sold in pet stores primarily as a diet for herbivorous aquarium fishes. All experiments took place at room temperature, approximately 23°C.

We isolated ten wild-collected snails from each of the two study populations in separate cups, collected egg masses, and reared the offspring to 3 mm shell length, approximately three weeks post-hatching, with weekly water change. These two sets of ten wild-conceived but laboratory-born sibships (A1-A10 and G1-G10) constituted the P generation for the four treatments (one experiment and three controls) we report here.

Each treatment was composed of ten replicates. Control A was a set of ten pairs of unrelated *Physa acuta* (A1 × A2, A2 × A3, . . . , A10 × A1). Control G was similarly constituted for *Physa gyrina* (G1 × G2, G2 × G3, . . .). The AG experiment was a set of ten cups of *P. acuta* paired with *P. gyrina* (AG1, AG2, . . . , AG10). The As control was a set of ten cups with isolated *P. acuta* snails (A1, A2, . . . , A10).

Each replicate received a water change and fresh food every seven days, at which time the sides of the cup were inspected for egg masses. If egg masses were present, we counted all embryos and transferred the adults to a fresh cup. Eggs were monitored until hatching, generally about two weeks, and all viable, crawling F_1 juveniles were counted. Replicates were terminated upon the mortality of either adult individual. For statistical analysis of fecundity (egg production) and F_1 viability (hatching success), week 1 was set separately for each treatment as the first week in which eggs were laid in three or more replicates. Fecundity and F_1 viability were subsequently recorded for ten weeks.

The central tendency of age at first reproduction was compared among the A, AG, and G treatments by dividing at the pooled median and testing the resulting 3 × 2 contingency table using chi-square. The AG experiment was compared to the As control in age at first reproduction using a similar median-based approach, although a Fisher's exact test was employed rather than chi-square, because of the former test's improved power. We compared the fecundity of the A, AG, and G treatments using two-way analysis of variance, with week and treatment the independent variables and embryos as the dependent variable (Statistica release 5.5, StatSoft 1994). Overall (ten-week) F_1 viability was compared among these treatments using analysis of covariance, with treatment the independent variable, viable hatchlings the dependent variable, and embryos the covariate. *Post hoc* tests were performed using Tukey's "highly significant difference" (HSD) tests for unequal sample sizes (Spjotvoll and Stoline 1973). A second ANOVA and a second ANCOVA were used to compare the fecundity and F_1 viability of treatment A to treatment As. Leading (pre-maturity) zeros were not included in any ANOVA or ANCOVA, nor were post-mortem zeros included, although internal zeros (i.e., reproductive failure by mature, apparently healthy snails) were analyzed.

To assess F_1 fertility in the AG, A , and G treatments, F_1 hatchlings were reared from each of three separate unrelated replicates to size 3 mm (AG1, AG2, AG3, A12, A34, A56, G12, G34, G56). These were paired across replicates within treatment in time series—one early pair from eggs laid around week 1, one middle pair produced around week 5, and one late pair produced around week 10. Each treatment thus yielded nine F_1 pairs. For example, treatment A yielded: A12 × A34 early, A12 × A34 middle, A12 × A34 late, A12 × A56 early, . . . , A34 × A56 late. Nine pairs were likewise constituted for treatments G and AG, and the total of 27 pairs of F_1 snails were reared to adulthood with weekly feeding and water change. We recorded the date at which embryos and viable F_2 hatchlings were produced by each pair.

A larger sample of 56 F_1 progeny from the AG1, AG2, and AG3 treatments was reared to 4-5 mm shell length, at which time they were frozen in 100/μl of tissue buffer for analysis by protein electrophoresis. Populations of *Physa acuta* and *Physa gyrina* share no alleles at six of the seven polymorphic allozyme loci routinely surveyed in our laboratory, including 6-phosphogluconic acid dehydrogenase (6Pgd) and isocitrate dehydrogenase (Isdh). We used horizontal starch gel electrophoresis in an aminopropylmorpholine pH 6 buffer system (Clayton and Tretiak 1972) to assess the allozyme phenotype of all putative F_1 hybrids at these two loci. Details regarding our electrophoretic methods, including a description of our equipment and recipes for stains and buffers, have been previously published (Dillon 1992, Dillon and Wethington 1995).

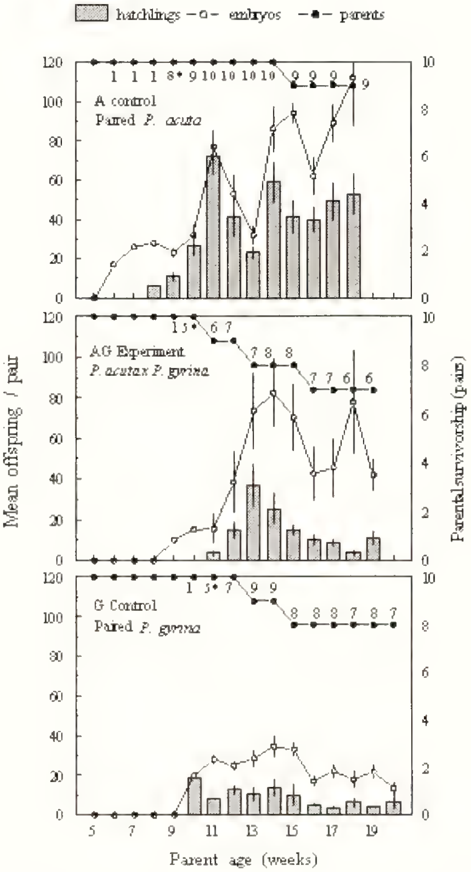

Figure 1. Production of embryos and viable hatchlings as a function of parental age (weeks post hatching) for ten pairs of *Physa acuta* (A control), ten pairs of *Physa gyrina* (G control) and ten pairs of *P. acuta* × *P. gyrina* (AG Experiment). The bars are standard errors of the mean. The number of reproducing pairs is given with parental survivorship (right axis). Asterisks* denote week 1 for analysis of variance.

RESULTS

Data on the production of F_1 progeny by the 10 pairs of parents in the A control, the G control, and the AG experiment are compared in Figure 1. The first pair of A parents laid eggs in week 6, although the median age at first reproduction was 9 weeks. Setting the ninth week of the treatment as week 1 for the purpose of analysis, over 10 weeks the mean weekly production of embryos was 61.9 per pair, and for hatchlings 39.1 per pair (63.0% viability). All 9 pairs of F_1 progeny successfully reproduced, laying eggs at a median age of 8 weeks that hatched to viable F_2 progeny a median of 2 weeks later.

The reproductive success of our G control of *Physa gyrina* was not as great as typically posted by *Physa acuta* under our culture conditions. Egg laying commenced at week 10 and reached a median between weeks 11 and 12 of the treatment. Setting week 11 to start, the mean fecundity over 10 weeks was only 21.2 embryos per pair per week, and the mean weekly F_1 hatchling yield was only 7.2 (33.5% viability). One F_1 pair was terminated by mortality, but the remaining 8 pairs laid viable eggs at a median age of 8 weeks that hatched to viable F_2 progeny at week 10.

Reproductive success in the AG experiment was generally intermediate between the A control and the G control. Egg laying began at week 9 and reached a median between weeks 10 and 11, yielding a mean fecundity of 36.4 per pair per week over 10 weeks. Hatchling production averaged only 9.3 per pair per week, for a 25.6% F_1 survival rate over that period. One pair of F_1 snails retained for testing ultimately proved sterile, but the remaining 8 pairs reproduced on the same schedule as the A and G controls: egg laying at a median of 8 weeks and viable F_2 hatchlings at week 10.

Our comparison of age at first reproduction in treatments A, AG, and G (Fig. 2) revealed a significant difference in central tendency ($\chi^2 = 9.02$, $p = 0.011$). Seven of the pairs of *Physa acuta* in control A reproduced before any of the pairs of *P. gyrina* in control G, with the AG experiment intermediate. Analysis of variance also uncovered a significant difference in fecundity (Table 1), HSD *post hoc* tests confirming that the A control produced significantly more embryos than the AG experiment ($p = 0.0149$), which yielded more embryos than the G control ($p = 0.0001$). Analysis of covariance (Fig. 3) returned a significant difference between treatments ($F = 16.8$, $p = 0.000$). HSD *post hoc* tests showed that the 63% F_1 viability posted by the A control was significantly greater ($p = 0.0001$) than either the 33% of the G control or the 26% of the AG experiment, which did not differ ($p = 0.8196$).

Protein electrophoretic analysis of F_1 progeny from the AG experiment revealed all viable offspring to be the products of self-fertilization by the *Physa acuta* parent. The 56 offspring we examined were distributed evenly across the ten weeks of reproduction in three replicates and were entirely homozygous for the *P. acuta* markers 6pgd100 and Isdh100 (Dillon and Wethington 1995). No hybrid progeny were recovered, nor did the *Physa gyrina* parent apparently reproduce successfully by self-fertilization.

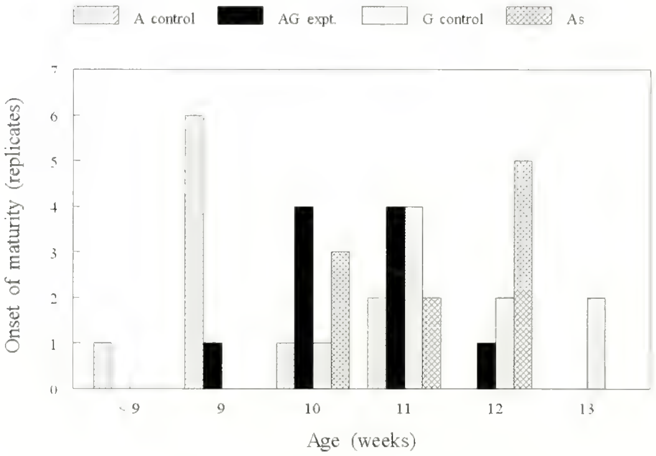

Figure 2. The number (of 10) replicates laying their first eggs as a function of parental age (weeks post-hatching) for pairs of *Physa acuta* (A control), pairs of *Physa gyrina* (G control), isolated individual *P. acuta* (As) and the *P. acuta* × *P. gyrina* experiment (AG).

Table 1. Results of analysis of variance comparing the fecundities measured over 10 weeks in the A control, the G control, and the AG experiment.

Effect	df effect	MS effect	df error	MS error	F	p-level
treatment	2	36,399	211	1,301	27.97	0.000
week	9	3,791	211	1,301	2.91	0.003
t × w	18	4,266	211	1,301	3.28	0.000

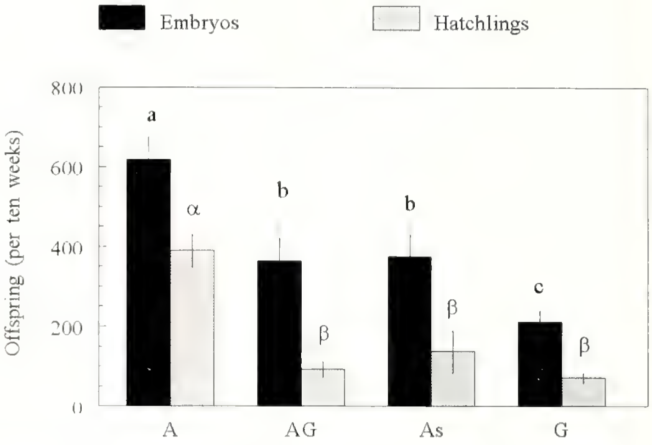

Figure 3. Total ten-week fecundity and yield of viable hatchlings (mean ± sem) for pairs of *Physa acuta* (A), pairs of *Physa gyrina* (G), the *P. acuta* × *P. gyrina* experiment (AG), and isolated individual *P. acuta* (As). Values significantly different by post hoc tests are designated with different lower case letters in Arabic for embryos or Greek for hatchlings.

Reproduction in the ten individual *Physa acuta* isolated for the As control is shown in Figure 4. Egg laying commenced at week 10 and reached a median at week 11. Taking week 10 as a start, the mean fecundity over ten weeks was 37.4 embryos per parent per week, yielding a weekly average of 13.9 hatchlings per parent for a 36.9% F_1 viability. Comparison of the AG experiment to the As control showed no difference in age at first reproduction (Fisher's exact probability = 0.65), fecundity ($p = 0.6795$, Table 2), or hatchling viability ($F = 0.82$, $p = 0.38$, Fig. 3).

DISCUSSION

The reproductive success we recorded for pairs of *Physa acuta* in these experiments (a median age of 9 weeks at first reproduction and 61.9 embryos per pair per week, 63% viability) was similar to that recorded for the Charleston population by Dillon *et al.* (2002). The present figures are lower than those reported for Charleston *P. acuta* by Wethington and Dillon (1997), but the 1997 experiments involved mated singletons (rather than pairs) and took place over the lifetime of the animals, rather than ten weeks.

Apparently our Virginia population of *Physa gyrina* is not as well-adapted to standard culture conditions as is

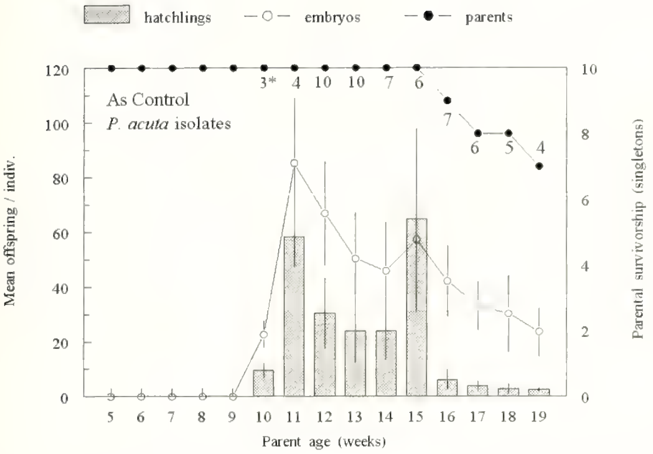

Figure 4. Production of embryos and viable hatchlings as a function of parental age (weeks post hatching) for ten individual *Physa acuta* reared in isolation (As control). The bars are standard errors of the mean. The number of reproducing individuals is given with parental survivorship (right axis). Asterisk* denotes week 1 for analysis of variance.

Table 2. Results of analysis of variance comparing the fecundities measured over 10 weeks in the As control and the AG experiment.

Effect	df effect	MS effect	df error	MS error	F	p-level
treatment	1	475	132	2,771	0.17	0.68
week	9	2,922	132	2,771	1.05	0.40
t × w	9	3,942	132	2,771	1.42	0.18

Physa acuta. Its reproductive record, which began at a median age of 11 weeks and featured only 21.2 embryos per pair per week with a 33.5% viability, was significantly below A control *P. acuta.* Indeed, the fecundity and F_1 viability posted by outcrossing pairs of *P. gyrina* was even below figures posted by our As self-fertilizing *P. acuta.*

Although copulation has been observed between *Physa acuta* and *Physa gyrina*, our results suggest that postmating reproductive isolation between the two species is complete. We cannot rule out the possibility that subviable F_1 hybrids (and pure *P. gyrina* as well) may have been born but were unable to compete with contemporaneous cohorts of pure *P. acuta.* In any case, all the progeny we recovered from ten weeks of joint culture were the products of self-fertilization by the *P. acuta* parent.

As has been previously reported (Wethington and Dillon 1997), individual *Physa acuta* isolated in our As control and forced to self-fertilize displayed delayed age at first reproduction (median age 11 weeks) and much-reduced F_1 viability (36.9%). There was no significant difference in the reproduction of As isolates and the self-fertilizing *P. acuta* cultured jointly with *Physa gyrina* (10.5 weeks, 36.4 embryos/week, 25.6% viability). Apparently, joint culture has neither a positive nor a negative effect on *P. acuta.* Culture with an individual *P. acuta* seems to be quite deleterious for *P. gyrina*, however, effectively shutting down whatever (relatively low) reproduction it would have otherwise achieved.

The results of this investigation offer no evidence of social facilitation between the two species of *Physa*. They do, however, contain an important cautionary message for future studies of postmating reproductive isolation in laboratory cultures of pulmonates. By none of the measures of fitness we employed here—age at first reproduction, fecundity, F_1 viability, and F_1 fertility—were the results of the AG experiment significantly depressed below either of the two controls. Our finding that the survivorship of the outcrossed F_1 progeny of *Physa gyrina* from the G control was not significantly greater than that of the selfed progeny of *Physa acuta* from the AG experiment was especially surprising. Had we not examined the allozyme phenotypes of the F_1 progeny via protein electrophoresis, and discovered that no hybrids were being produced, the reproductive isolation displayed between species as different as *P. acuta* and *P. gyrina* might have been missed entirely.

ACKNOWLEDGMENTS

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High levels of mitochondrial DNA sequence divergence in isolated populations of freshwater snails of the genus *Goniobasis* Lea, 1862*

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Abstract: In addition to their utility for phylogenetic reconstruction, mitochondrial sequence data have increasingly been applied to studies of species-level systematics. We amplified and sequenced a 709 bp fragment of the mitochondrial gene encoding cytochrome oxidase 1 and an approximately 530 bp fragment of the ribosomal large subunit (16S) gene for three individuals from each of three populations representing geographic races of the well-studied freshwater "prosobranch" snail *Goniobasis proxima*. By comparing intraspecific divergence to divergence in these same genes among *G. proxima* and the related *Goniobasis semicarinata* and *Goniobasis catenaria*, our purpose was to calibrate mitochondrial sequence data for application in future systematic studies of isolated, poorly-mobile molluscan populations in which genetic relationships may be less well understood. We identified four distinct haplotypes in the nine mitochondrial genomes of *G. proxima* amplified for each gene fragment, with a maximum likelihood sequence difference of 8.6%-16.9% for CO1 and 5.7%-18.7% for 16S. These levels of intraspecific divergence overlapped extensively with interspecific maximum likelihood differences, which ranged from 11.4%-17.7% for CO1 and 9.5%-16.5% for 16S. The extreme fragmentation that typically characterizes the population structure of freshwater gastropods, together with the ability of such populations to reach large size and great age, must be taken into consideration before systematic inference can be made on the basis of sequence divergence for these genes.

Keywords: 16S, CO1, *Elimia*, Virginia, Carolina

All major invertebrate taxa include poorly known groups in which the relationships between species are not clear. In freshwater and terrestrial molluscs, for example, species ranges may be fragmented into isolated populations and variation in shell morphology and other traditional characters may be negligible or subject to phenotypic plasticity. Thus as the tools of molecular genetics have become more accessible, malacologists have turned to DNA sequence data as a source of evidence by which biological species may be distinguished.

Before any new measurement tool can be employed efficiently, however, it must be calibrated. Levels of DNA sequence divergence should first be examined within and among populations for which specific relationships have previously been established by breeding studies or similarly direct means. If molecular data are to achieve ideal utility as criteria for species recognition, the maximum levels of sequence divergence among populations known to be conspecific should be less than the minimum sequence divergence between known, closely related species.

Among the most commonly sequenced genes in studies of molluscan population divergence are the mitochondrial genes encoding cytochrome oxidase I (CO1) and the large ribosomal subunit (16S). Sequence variation for the CO1 gene distinguishes unambiguously among species of the marine vesicomyid clams (Baco *et al.* 1999), the freshwater bivalve genera *Lasmigona* Rafinesque, 1831 (King *et al.* 1999) and *Corbicula* Megerle von Mühlfeld, 1811 (Renard *et al.* 2000), and the marine gastropod genera *Crepidula* Lamarck, 1799 (Collin 2000), *Notoacmaea* (Simison and Lindberg 1999), and *Hydrobia* Hartmann, 1821 (Wilke and Davis 2000, Wilke *et al.* 2000). Sequence variation for the 16S gene effectively discriminates among species in the marine bivalve genera *Ostrea* Linnaeus, 1758 (Ó Foighil *et al.* 1995, 1998, Jozefowicz and Ó Foighil 1998), and *Mercenaria* Schumacher, 1817 (Ó Foighil *et al.* 1996), and in the freshwater bivalve genera *Amblema* Rafinesque, 1820 (Mulvey *et al.* 1997) and *Dreissena* van Beneden, 1835 (Stepien *et al.* 1999). The 16S gene has also proven useful to distinguish species of land snails in the genera *Candidula* Kobelt, 1871 (Pfenninger and Magnin 2001), *Discus* Fitzinger, 1833 (Ross 1999) and *Cepaea* Held, 1837 (Thomaz *et al.* 1996).

In some situations, however, the level of sequence divergence within molluscan species has been found to exceed divergence among species. In the special case of doubly-uniparental inheritance, the male and female mitochondrial genomes within populations of *Mytilus* spp. and *Anodonta* spp. have diverged more than between same-sex comparisons of valid species (Rawson and Hilbish 1995, Hoeh *et al.* 1996). Sequence divergence among conspecific populations of the land snail *Mandarina* sp. from remote Pacific islands has proceeded to the extent that between-species variance seems to have been swamped (Chiba 1999).

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* From the symposium "The Biology and Conservation of Freshwater Gastropods" presented at the annual meeting of the American Malacological Society, held 3-7 August 2002 in Charleston, South Carolina, USA.

We are aware of four prior works comparing the levels of DNA sequence divergence within and among conspecific populations of freshwater gastropods to divergence between related species. In three of these cases, researchers have reported an overlap between the maximum levels of divergence within species and the minimum divergence between species. In the pleurocerid fauna of Alabama, Lydeard *et al.* (1998) reported that 16S divergence ranged from 0% to 3.93% within species of *Goniobasis* Lea, 1862 (or *Elimia* H. and A. Adams, 1854) and from 0.3% to 11.08% between species. Populations representing different subspecies of the oriental pomatiopsid *Oncomelania hupensis* (Gredler, 1881) may show CO1 sequence differences exceeding those reported between the related pomatiopsid genera *Gammatricula* Davis and Liu, 1990 and *Tricula* Benson, 1843 (Davis *et al.* 1998). Hershler *et al.* (1999b) reported that the CO1 divergence between two Death Valley populations of the hydrobiid *Tryonia variegata* Hershler and Sada, 1987 was equal to or greater than the level observed in most other comparisons among eight species of *Tryonia* Stimpson, 1865. The subsequent results of Hershler *et al.* (1999a) on a larger sample of hydrobiids from the American southwest seemed to cast doubt on previous assumptions regarding specific relationships in this group. In any case, it is possible that the evolution of DNA sequences in freshwater gastropods may be more similar to that described by Chiba (1999) for island populations of the land snail *Mandarina* sp. than to the bivalves, marine gastropods, or even most terrestrial gastropods that have attracted the bulk of previous study.

At the level of its population genetics, the pleurocerid *Goniobasis proxima* (Say, 1825) is among the best known of all freshwater gastropods. The purpose of this paper was to assess sequence variation for the mitochondrial CO1 and 16S genes within and among populations of *G. proxima* and compare the intraspecific values obtained to interspecific values from two other well-characterized *Goniobasis* species known to be related. Our purpose is to confirm the small body of previously published evidence suggesting that populations of freshwater snails may be so old, large, and isolated that intrapopulation sequence divergence is liable to swamp interpopulation sequence divergence in two of the mitochondrial genes most commonly examined by malacologists using the tools of molecular genetics.

The Pleuroceridae is a holarctic family of freshwater "prosobranch" gastropods that has diversified extensively in the rivers and streams of the American southeast. Populations are perennial and may reach great densities, locally hundreds per square meter. Reproduction is entirely sexual, as far as is known. The biology of the Pleuroceridae has been reviewed by Dillon (2000). *Goniobasis proxima* is a common pleurocerid inhabitant of small softwater streams in the piedmont and mountains from southern Virginia to northern Georgia, on both sides of the eastern continental divide. Populations are isolated both by intervening mountain ranges and by larger rivers, to which the snail does not seem adapted. A sample of 25 populations from a 20,000 km^2 area straddling the borders of Virginia, North Carolina, and Tennessee showed extreme divergence in both morphology and allozyme frequencies; some pairs of populations sharing no alleles at multiple enzyme loci (Dillon 1984a). Three races of *G. proxima* have been recognized (A, B, and C) on the basis of shell morphology and allozyme divergence inhabiting different parts of the range (Dillon and Davis 1980, Dillon 1984b). Transplant experiments (Dillon 1988a) and artificial introductions (Dillon 1986) have, however, uncovered no evidence of reproductive isolation among any of these populations.

As genetically diverse as *Goniobasis proxima* may be, its levels of interpopulation divergence do not approach those recorded among other well-characterized species, such as *Goniobasis semicarinata* (Say, 1829) (Dillon and Davis 1980) or *Goniobasis catenaria* (Say, 1822) (Dillon and Reed 2002). *Goniobasis semicarinata* is primarily an inhabitant of the American interior, ranging through Ohio, Indiana, and Kentucky. Its biology is similar to that of *G. proxima*, although it bears a heavier shell and is restricted to harder water and lower elevations. The southern limit of *G. semicarinata* contacts the northern border of the range of *G. proxima* in the New River drainage of Virginia. *Goniobasis catenaria* inhabits streams and rivers on the southern and eastern borders of the *G. proxima* range (Dillon and Keferl 2000). There are several subspecies, one of which (*G. catenaria dislocata*) bears a shell distinguishable from that of *G. proxima* only by faint axial costae. The 2n = 36 karyotype of *G. catenaria* is not strikingly different from the 2n = 34 karyotype of *G. proxima* (Dillon 1989, 1991), but there is no evidence of hybridization between the two species, even in close contact (Dillon and Reed 2002). So analyzed together, the *Goniobasis* fauna of the southeastern United States would seem an excellent model upon which to gauge the utility of mitochondrial sequence data for species discrimination in freshwater gastropods, and perhaps in poorly-mobile freshwater invertebrates more generally.

METHODS

Study populations

Although separated by less than 120 km over land, our three populations of *Goniobasis proxima* shared no freshwater connection (Fig. 1). Our Race A sample came from a tributary of the Yadkin River, which drains south to the Atlantic through the Pee Dee system, our sample of Race B was from a tributary of the New River, flowing west to the Mississippi through the Ohio River system, and our sample of Race C was from a small tributary of the Dan River, flowing east to the Atlantic through the Roanoke River system. Our sample of *Goniobasis semicarinata* was collected from a small tributary of the New River only 50 km east of our *G. proxima* Race C. Our *Goniobasis catenaria* came from a tributary of the Santee River, which flows through South Carolina to the Atlantic approximately 350-400 km south of the other four populations.

Detailed locality data are as follows: *Goniobasis proxima* Race A-Naked Creek at NC 1154 bridge, 5.2 km N of Furguson, Wilkes Co., NC. *Goniobasis proxima* Race B-Cripple Creek at Va 671 bridge, 3.7 km E of Cedar Springs, Wythe Co., VA. *Goniobasis proxima* race C-Nicholas Creek at Va 623 bridge, 5.2 km SW of Ferrum, Franklin Co., VA. *Goniobasis semicarinata*-Little Pine Run at Va 100 bridge, 12 km S of Pulaski, Pulaski Co, VA. *Goniobasis catenaria dislocata*-the head of Chapel Branch, Santee, Orangeburg Co., SC. Allozyme data and maps locating these populations have been published as follows: Race A is "Yad" of Dillon and Davis (1980) and Dillon and Reed (2002) or "Yad1" of Dillon (1984a, 1988b). Race B is "Crip" of Dillon and Davis (1980) and Dillon (1984a, 1988a). Race C is "Phlp" of Dillon (1984a). Our *Goniobasis semicarinata* is population "Pine" of Dillon and Davis (1980) and our *G. catenaria dislocata* is population "Sant" of Dillon and Reed (2002).

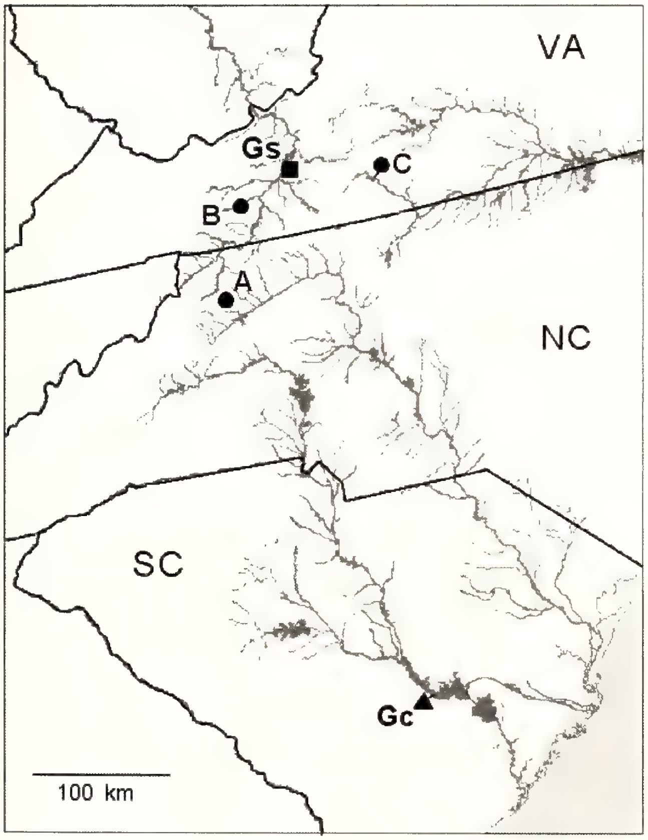

Figure 1. A portion of the southeastern United States, showing drainage relationships among sample sites. The circles are populations of *Goniobasis proxima* (A, B, C), the square is a population of *Goniobasis semicarinata* (Gs), and the triangle is a population of *Goniobasis catenaria* (Gc).

We analyzed three individuals from each race of *G. proxima* and one individual from each of the other two species. Thus a total of 11 fragments were amplified and sequenced for each of the two mitochondrial genes examined here.

Laboratory methods

Total cellular DNA was obtained using either fresh or previously frozen samples of whole buccal mass (~20 mg). Following proteinase K digestion of the tissue, the DNA was extracted using either a DNeasy Tissue Kit (Qiagen) or by two phenol and two chloroform extractions followed by ethanol precipitation. The optimum DNA concentration for PCR of each of the samples was determined empirically.

We amplified a 709 bp fragment of the mitochondrial cytochrome oxidase I gene using the "universal" CO1 primers of Folmer *et al.* (1994): 5′-ggtcaacaaatcataaagatattgg-3′ and 5′-taaacttcagggtgaccaaaaaatca-3′. Our 525-532 bp fragment from the 5′ half of the 16S mitochondrial rDNA gene was amplified using primers "SNL002" and "SNL448" of Lydeard *et al.* (1997, 1998), the former trimmed slightly to afford a better match of annealing temperatures: 5′-aaatgattatgctaccttt-3′ and 5′-gaaatttcattcgcactag-3′. The primer "16sar-L" (or L2510) commonly used as a starting point to amplify the 3′ half of the mitochondrial 16S gene (Palumbi *et al.* 1991) is encountered around bases 410-430 of the sequences we determined in the present study. Lydeard *et al.* (1998) reported that the 5′ half of the pleurocerid 16S gene shows greater variability than the 3′ half more usually sequenced by other workers.

A typical amplification reaction of 50 μL contained 1.5 μL of DNA (the optimum amounts generally ranged between 50 and 200 ng, determined empirically), 100 mM Tris (pH 9), 50 mM KCl, 1.5 units of Taq DNA polymerase, 150 μM of each of the four deoxynucleotide triphosphates, and 0.2 μM of each primer. PCR amplification was by a 15 min 95°C activation step followed by 30 cycles, each consisting of 45 sec at 94°C, 1 min at 45°C, and 1 min at 72°C. Upon completion of the 30 cycles a 10 min 72°C incubation was performed to extend uncompleted strands. Amplification of fragments of the expected size was verified by agarose gel electrophoresis. The PCR-amplified DNA was prepared for sequencing using a QIAquick PCR purification kit (Qiagen). Cycle sequencing was performed by the Medical University of South Carolina Biotechnology Resource Laboratory. The PCR product was sequenced twice for both strands.

Analysis

Our initial alignments of the four sequence fragments obtained for each individual were performed using the "Web align" feature of Biowire Jellyfish (version 1.5, Biowire.com) with default settings. Final alignments (between individuals) were performed online with program *blastn* through the "Blast two sequences" utility available from the National Center for Biotechnology Information (Tatusova and Madden 1999, NCBI 2003). The apparently high frequency of indels in our 16S data prompted us to lower the gap opening penalty from 10 to 2 and the gap extension penalty from 2 to 1. The strong A/T bias also observable in our 16S data prompted us to de-select the low complexity filter.

We translated our CO1 sequence fragments with the invertebrate mitochondrial code and a +3 lag using Biowire Jellyfish, then aligned the resulting amino acid sequences pairwise with the *blastp* program, also available online through the NCBI "Blast two sequences" utility (NCBI 2003). All *blastp* parameters were set to default, except that the low complexity filter was de-selected.

We recorded the simple percent nucleotide difference ("p distance") between each unique pair of sequences as one minus the identity returned by the pairwise BLAST utility, extending through the entire (unvaried) primer region on each end. Where indels had apparently yielded two sequences of different lengths, the length of the larger sequence served as denominator in the calculation of identity. Thus the impact of each indel was weighted by its length.

We also calculated maximum likelihood (ML) distances among our sequence fragments using the DNADIST program available in PHYLIP (version 3.573c, Felsenstein 1995). Both the base composition frequencies and transition:transversion ratios were determined empirically. A single joint analysis was performed for the CO1 data. Because indels are scored as missing data (rather than as mismatches) in the calculation of ML distances, however, their accumulated effects across the diverse 16S sequences we ultimately obtained would have resulted in substantial loss of data upon multiple alignment. We therefore elected to calculate ML distances among 16S sequences pairwise in multiple separate runs, rather than in a single joint analysis.

For parsimony analysis, we performed a conventional multiple alignment of our 16S sequences using BioEdit version 5.0.9 (Hall 1999) and concatenated each 16S sequence (elongated by multiple insertions) to its corresponding CO1 sequence. We then analyzed the combined data set using PAUP* version 4.0b10 (Swofford 2002), setting *Goniobasis semicarinata* as root, *Goniobasis catenaria* as root, and *semicarinata* + *catenaria* as root, with 1,000 bootstrap replicates.

RESULTS

Results for the CO1 and the 16S genes were similar in many respects. All three individuals of *Goniobasis proxima* from population A yielded identical CO1 and 16S haplotypes, as did all three individuals from population B. Population C yielded two strikingly different CO1 haplotypes and two strikingly different 16S haplotypes. For both genes, the haplotype carried by two individuals was designated "C1" and the haplotype carried by the third snail was designated "C2." The two other species, *Goniobasis semicarinata* and *Goniobasis catenaria*, also yielded distinct haplotypes, resulting in six unique sequence fragments for each gene. The total of 12 unique sequences has been entered in GenBank, accession numbers AY063464-AY063475.

The total length, from the 5′ beginning of the first primer to the 3′ end of the second primer, was 709 bp for all six unique CO1 sequence fragments. Table 1 shows that the simple uncorrected nucleotide difference between the four sequences of *Goniobasis proxima* ranged from 8.0% between populations A and B to 14.7% between the two sequences identified in population C. This translated to an amino acid difference of 1.3%-5.5%. Interspecific divergence was not strikingly different from intraspecific divergence, evaluated at the maximum. The uncorrected difference between *Goniobasis semicarinata* and the other species, and between *Goniobasis catenaria* and the other species, ranged from 10.2%-15.2% as nucleotides or 0.4%-5.1% as amino acids.

Combined over the six unique CO1 sequences we obtained, the base frequencies were 24%A, 19%C, 20%G, and

Table 1. Comparisons of six mitochondrial CO1 sequence fragments amplified from three species of *Goniobasis*. Above the diagonal are the percent differences (p distances) of nucleotide bases (709 in the denominator, including both primers) and below the diagonal are percent amino acid differences (235 in the denominator).

	A	B	C1	C2	*G.s.*	*G.c.*
G. proxima A		8.0	14.0	14.1	11.7	10.9
G. proxima B	4.3		12.7	14.5	10.2	10.3
G. proxima C1	2.1	5.5		14.7	13.5	15.2
G. proxima C2	1.7	5.1	1.3		13.5	14.4
G. semicarinata	0.4	3.8	1.7	1.3		12.1
G. catenaria	0.9	5.1	3.0	2.1	1.3	

37%T. Pairwise transition : transversion (Ts : Tv) ratios are shown in Table 2, the overall empirical ratio being 4.14. Corrected by the base frequencies and the Ts : Tv ratio, the six sequences are arranged by their maximum likelihood distances in Fig. 2.

The distance from the 5′ end of the leading primer to the 3′ end of the trailing primer for the 16S fragment amplified in this study ranged from 524-532 bp for the six unique sequences we obtained. Table 3 shows that uncorrected sequence differences ranged from 6.1%-17.1% among the populations of *Goniobasis proxima*, with 1-4 indels apparent for each comparison. Evaluated at the maximum, this is again not strikingly different from the levels of divergence between species, which ranged from 9.3%-17.9%.

Variable bases did not seem equally distributed across the approximately 530 bases of the 16S fragment we amplified, but rather seemed localized, as one might expect from the stem-and-loop structures assumed by ribosomal subunits. Apparent Ts : Tv ratios were generally low, approaching unity in several instances (Table 2). Across all six unique 16S sequence fragments (3,170 bases) the base frequencies were 35% A, 36% T, 13% C, and 16% G. Corrected by base composition and pairwise Ts : Tv ratios, the sequences are diagramed by their maximum likelihood distances in Fig. 3.

Multiple alignment elongated the joint 16S product to 545 bases by multiple insertion, which when concatenated with the 709 CO1 bases yielded a combined sequence of 1,254 characters. Of these 928 were constant, 207 were parsimony-uninformative, and 119 were parsimony-informative. Phylogenetic analysis with both *Goniobasis semicarinata* and *Goniobasis catenaria* specified as roots returned single trees of length 478 (CI = 0.8159, HI = 0.1841), both depicting *Goniobasis proxima* as paraphyletic. The third analysis, combining *G. semicarinata* and *G. catenaria*, could not be rooted such that the outgroup was monophyletic, and collapsed to yield the single tree rooted by *G. semicarinata* alone (Fig. 4).

Table 2. Apparent transition : transversion ratios in comparisons of six mitochondrial sequence fragments amplified from 3 species of the genus *Goniobasis*. Data for the CO1 gene are above the diagonal, and those for the 16S gene are given below.

	A	B	C1	C2	*G.s.*	*G.c.*
G. proxima A		16.7	3.50	3.50	4.57	3.81
G. proxima B	6.00		3.05	3.86	3.80	3.50
G. proxima C1	3.86	8.00		2.52	2.25	3.12
G. proxima C2	2.35	2.33	2.15		2.56	2.33
G. semicarinata	3.42	2.83	2.38	1.31		3.05
G. catenaria	4.18	3.46	2.86	1.18	1.29	

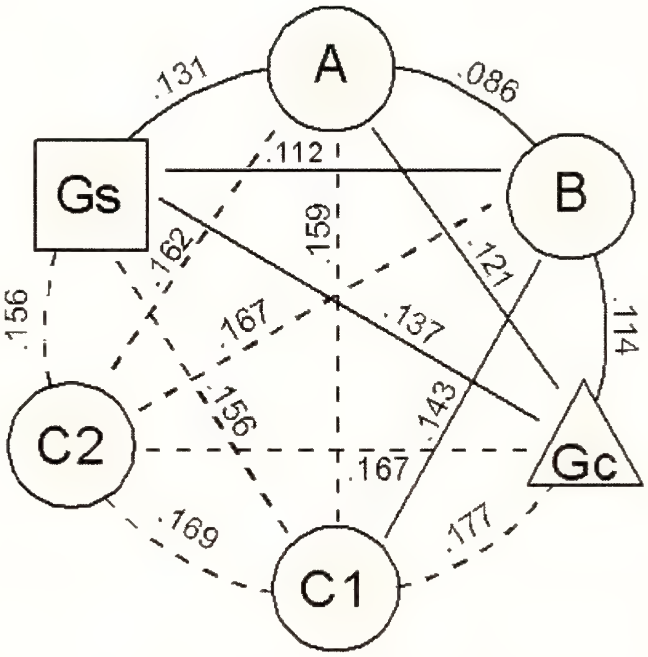

Figure 2. The six unique mitochondrial CO1 sequence fragments diagramed by their maximum likelihood distances. The circles are *Goniobasis proxima* (A, B, C1, C2), the square is *Goniobasis semicarinata* (Gs), and the triangle is *Goniobasis catenaria* (Gc). Thick segments join haplotypes that are less than 10% different, thin segments connect haplotypes ranging from 10%-15% different, and dashed segments join haplotypes of greater than 15% maximum-likelihood distance.

DISCUSSION

The level of sequence divergence we observed within and among conspecific populations of *Goniobasis proxima* was exceptionally high. A review of the molluscan literature suggests that intraspecific divergence in either of the genes we examined here typically ranges no higher than 5%. This generalization holds true for marine gastropods (Simison and Lindberg 1999, Collin 2000, Hamm and Burton 2000, Wilding *et al.* 2000, Wilke and Davis 2000), marine bivalves (Geller *et al.* 1993, Ó Foighil *et al.* 1996, Chase *et al.* 1998, Ó Foighil *et al.* 1998, Baco *et al.* 1999), and freshwater bivalves (King *et al.* 1999, Stepien *et al.* 1999, Renard *et al.* 2000). A notable exception occurs in the sex-specific haplotypes of certain bivalves, which may differ by as much as 30% (Hoeh *et al.* 1996, 1997). Levels of sequence divergence among conspecific populations of land snail are also generally reported to reach maxima higher than the 5% typical for most molluscs: 5.3% in *Partulina* Pfeiffer, 1854 (Thacker and Hadfield 2000), 8% in *Candidula* (Pfenninger and Magnin 2001), 8.4% in *Discus* (Ross 1999), 9.5% in *Euhadra* Pilsbry, 1890

Table 3. Comparisons of six mitochondrial 16S sequence fragments amplified from three species of *Goniobasis.* The diagonal gives the sequence length, including both primer regions. Above the diagonal are percent nucleotide differences (p distances), where the denominator is the sum of the sequence length and its corresponding apparent number of indel bases. Below the diagonal is the number of indels, recorded as the apparent number of deletions in the column sequence (total bases) over the number of deletions in the row sequence (total bases).

	A	B	C1	C2	*G.s.*	*G.c.*
G. proxima A	528	6.1	7.4	17.1	11.2	12.5
G. proxima B	0/3(3)	525	6.2	16.4	9.3	12.9
G. proxima C1	1(2)/0	3(5)/0	530	14.5	9.8	12.8
G. proxima C2	3(5)/2(2)	4(6)/0	4(7)/2(6)	531	14.7	17.9
G. semicarinata	0/2(4)	1(1)/2(2)	0/3(6)	1(1)/4(8)	524	11.8
G. catenaria	2(7)/3(3)	3(8)/1(1)	4(8)/4(6)	8(12)/3(11)	3(8)/0	532

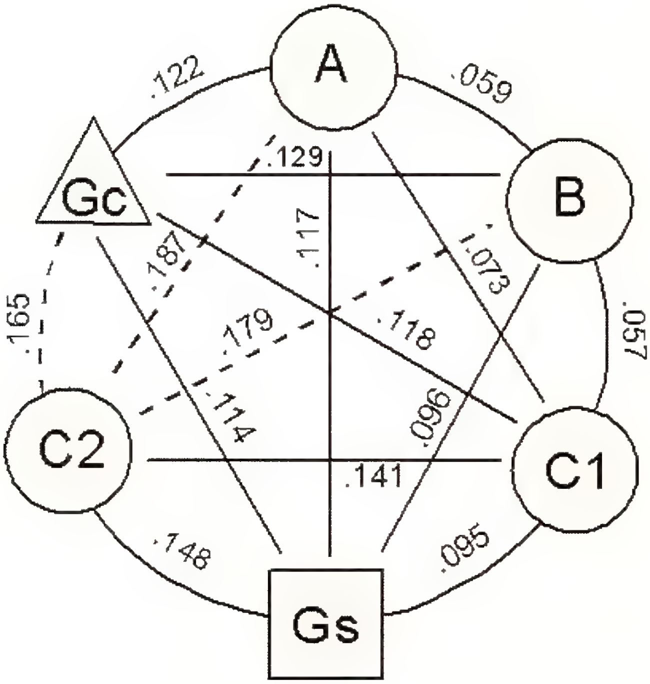

Figure 3. The six unique mitochondrial 16S sequence fragments diagramed by their maximum likelihood distances. The circles are *Goniobasis proxima* (A, B, C1, C2), the square is *Goniobasis semicarinata* (Gs), and the triangle is *Goniobasis catenaria* (Gc). Thick segments join haplotypes that are less than 10% different, thin segments connect haplotypes ranging from 10%-15% different, and dashed segments join haplotypes of greater than 15% maximum-likelihood distance.

(Hayashi and Chiba 2000), 11.1% in *Helix* Linné, 1758 (Guiller *et al.* 2001), 12.9% in *Cepaea* (Thomaz *et al.* 1996), 13% in *Arianta* Leach *in* Turton, 1831 (Haase *et al.* 2003) and 18.7% in *Mandarina* (Chiba 1999).

It is difficult to generalize regarding the levels of sequence divergence previously reported among conspecific populations of freshwater gastropods. Lydeard *et al.* (1998) compared 8 individual *Goniobasis* (or *Elimia*) *carinocostata* (Lea, 1845) from five sites and obtained a maximum 16S sequence divergence of 3.9%. The maximum CO1 divergence among conspecific individuals of *Tryonia* from Death Valley was 5.2% (Hershler *et al.* 1999b). Within subspecies of *Oncomelania hupensis*, the maximum CO1 divergence seemed to average around 2.1% (Davis *et al.* 1999), while maxima between the subspecies reached 14.2-15.3% (Davis *et al.* 1998).

In *Goniobasis proxima*, we have discovered maximum divergences of p = 14.7% or ML = 16.9% for the CO1 gene and p = 17.1% or ML = 18.7% for the 16S gene. These rank among the highest intraspecific value for sequence divergence yet reported for molluscs. Thomaz *et al.* (1996) suggested four (overlapping) explanations for the high levels of sequence divergence they observed among populations of the land snail *Cepaea nemoralis* (Linné, 1758): a large effective population size, fragmentation of the range into isolated demes, disruptive selection, and a systemically higher rate of mitochondrial evolution. Of these four, the factor most conspicuously shared by the land and freshwater snails (but not by marine molluscs or by bivalves generally) is population fragmentation. Our three populations of *G. proxima* shared no connection through water and may have been isolated for millions of years.

Our sample of three Race C snails included a pair of strikingly divergent haplotypes for both the genes we examined. The population from which these snails were drawn ("Phlp" of Dillon 1984a) is homogeneous both in morphology and in gene frequency at seven enzyme-encoding nuclear genes. Apparently, conspecific pleurocerids sampled from adjacent rocks may show more mitochondrial DNA sequence divergence than verifiably distinct species isolated by 400 km overland. Data addressing the possible existence of intermediate forms between these two diverse mitochondrial haplotypes in the Phlp population would cast light on whether such great genetic diversity may have evolved *in situ* or might reflect the admixture of two previously isolated populations.

The levels of sequence divergence we observed within and among populations of *Goniobasis proxima* exceeded their (interspecific) divergence with *Goniobasis semicarinata* and *Goniobasis catenaria* in many cases. For the CO1 gene, haplotypes A and B were more similar to *G. semicarinata* or

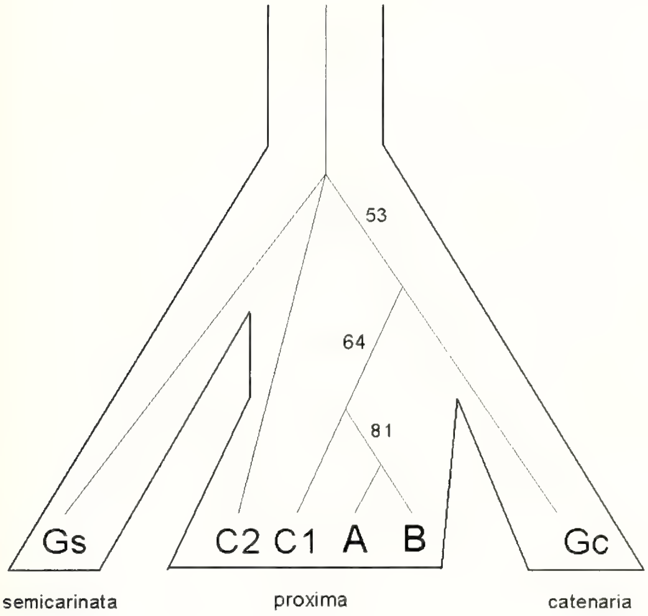

Figure 4. The single most parsimonious tree (CI = 0.816, HI = 0.184) returned by phylogenetic analysis where *Goniobasis semicarinata* was specified as root. Bootstrap values (percent of 1,000 replicates) are indicated at the nodes.

G. catenaria than to either C1 or C2, regardless of the metric employed (Table 1, Fig. 2). For the 16S gene, haplotype C2 was so strikingly distinct as to render all the other five haplotypes neighbors by comparison (Table 3, Fig. 3).

Phylogenetic analysis of the combined data set under the parsimony criterion suggested that *Goniobasis proxima* is paraphyletic with respect to either of its congeners in the American southeast. This may be a consequence of "lineage sorting" as depicted in Fig. 4 (Takahata and Nei 1985, Rosenberg 2003). The bootstrap support for most of the branches in the phylogeny was not high, however, and the tree topology may reflect more noise than signal.

There is some evidence that the sites available for variation in these genes may be approaching saturation in this sample of populations of *Goniobasis*. Haplotypes A and B appeared to be the most similar by almost all measures, and displayed an exceptionally high Ts : Tv ratio (Table 2). Omitting the A/B comparison for CO1, the overall average Ts : Tv ratio across the six populations droped from 4.14 to 3.24. The relationship between B and C1 was also apparently close (judging from 16S data) and characterized by a high Ts : Tv ratio. Setting aside these two individual comparisons, however, the Ts : Tv ratios we obtained were generally less than 4 : 1. It is interesting to note that the greatest difference in CO1 amino acid sequence (5.5%) was posted between *Goniobasis proxima* B and C1, which the maximum likelihood analysis of 16S sequence data suggested as the most similar pair of populations. Our observation that interspecific differences in amino acid sequence were strikingly lower than intraspecific differences in most cases (Table 1) further suggests that sequence divergence may be approaching saturation in these highly isolated populations of freshwater snails.

Under such circumstances, systematic inference must be made with care. The existence of a high level of sequence divergence can apparently be interpreted as little evidence that a pair of freshwater snail populations is specifically distinct. The only other pleurocerid populations for which data are available on both gene frequencies at nuclear loci and mitochondrial sequence divergence are the *Leptoxis* spp. of Alabama. Lydeard *et al.* (1997) reported up to 19.4% sequence divergence for the 16S gene among single individuals of three nominal species: *Leptoxis ampla* (Anthony, 1855), *Leptoxis taeniata* (Conrad, 1834), and *Leptoxis picta* (Conrad, 1834). These results appeared incompatible with a much larger data set on gene frequencies at nine enzyme-encoding loci in six populations (30 individuals per population), which suggested that the three nominal species might be conspecific (Dillon and Lydeard 1998). It now seems apparent that an uncorrected sequence difference as high as 19% for the 16S gene does not necessarily contradict the conspecific hypothesis for pleurocerid populations.

An observation of low levels of divergence may constitute some evidence that a pair of populations is conspecific, however. In addition to their data on the three individuals of *Leptoxis* spp. noted above, Lydeard and his colleagues (Lydeard *et al.* 1997, 1998, Holznagel and Lydeard 2000) have reported sequence data from the 16S rDNA gene for over 30 species (representing five genera) of North American pleurocerid snails. Their sampling effort has focused on the Mobile Basin of Alabama because of its putatively high pleurocerid diversity, and has been directed toward elucidating higher-level evolutionary relationships. Like most of the American freshwater gastropod fauna, however, the taxonomy of the Alabama Pleuroceridae predates the modern synthesis, being based almost entirely upon minor attributes of the shell. The maximum divergence among the individual snails representing seven nominal species of Alabama *Goniobasis* sequenced by Lydeard *et al.* (1997) was only 7.89% (uncorrected), with many pairwise values less than 2%. A critical re-examination of the biological species of pleurocerid snails in the Mobile Basin, and throughout most of North America, is long overdue.

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Species composition and geographic distribution of Virginia's freshwater gastropod fauna: A review using historical records*

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Abstract: Survey data from electronic databases and the literature were used to summarize knowledge of the composition and geographic distribution of Virginia's freshwater gastropod fauna. After excluding records likely based on misidentifications, we concluded that 53 species of freshwater gastropods occur in Virginia now or historically. A map and/or narrative description of statewide distribution was produced for each species. Several species appeared to be restricted to a few sites and highly endangered, including the hydrobiids *Fontigens bottimeri, Fontigens morrisoni, Holsingeria unthankensis,* and *Holsingeria* sp. 1. Absence of recent records for the hydrobiid *Somatogyrus virginicus,* the pomatiopsid *Pomatiopsis cincinnatiensis,* the pleurocerids *Elimia arachnoidea* and *Pleurocera gradata,* and the lymnaeid *Stagnicola neopalustris* indicated these species might also be imperiled if not already extirpated from Virginia. Although we have a good understanding of distributions of *Fontigens* spp., *Holsingeria* spp., and of several river-dwelling pleurocerids in southwest Virginia, other species and geographic regions (e. g., eastern shore and Big Sandy River drainage) are undersurveyed. We provide data to assist in designing surveys to fill these knowledge gaps and to monitor temporal changes in species' distributions. Comparisons of historic and future data from field surveys will facilitate protection and management of endangered species by providing evidence of restricted or shrinking geographic ranges.

Key words: Macroinvertebrates, biogeography, endangered species, snails

In 1817, Thomas Say published the first descriptions of species of freshwater gastropods in North America (Say 1817, Martin 1999). Although our understanding of this continent's freshwater gastropod fauna has advanced since that time, large gaps remain. For example, taxonomic confusion precludes accurate estimates of numbers of species inhabiting North America and makes it difficult to determine the geographic distribution, environmental requirements, ecological importance, and conservation status for many taxa (Neves *et al.* 1997). Additionally, many species are thought to have experienced dramatic population declines, but quantitative evidence to confirm this is rarely available. It has been determined, however, that at least 42 species have become extinct following European settlement of North America, and living specimens of several other species have not been seen in nearly a century (Neves 1991, Neves *et al.* 1997, Bogan 1998). Clearly, the lack of attention to freshwater gastropods has been costly.

Field surveys provide critical evidence of changes in freshwater gastropod assemblages, including population declines and shrinking or restricted geographic ranges. Although large quantities of survey data exist for many North American species, many data are scattered among museum collections and unpublished and published literature that are difficult to obtain. These data must be summarized and disseminated to gain a complete understanding of the distribution and conservation status of our freshwater gastropods and to identify geographic regions requiring additional surveys.

We reviewed and summarized data from field surveys in electronic databases and the literature to describe the species composition and geographic distribution of freshwater gastropods in Virginia, U.S.A. Geographic information associated with collection records was used to produce maps and narrative descriptions of distributions of species inhabiting Virginia now or historically. This is the first comprehensive review of Virginia's freshwater gastropod fauna in 30 years. Beetle (1973a) used museum collections to construct a checklist of Virginian species with names of counties or cities, but not specific locality data. Burch (1950) and Burch (1952) published regional species checklists for the James River Basin and Hanover County. Other surveys in Virginia concentrated on specific drainage basins or taxonomic groups (Clench and Boss 1967, Stansbery and Clench 1974a, Stansbery and Clench 1974b, Stansbery and Clench 1977, Dillon and Benfield 1982, Hershler *et al.* 1990).

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METHODS

Study area

Virginia extends from 36°30′N to 39°30′N in latitude, and from 75°13′W to 83°40′W in longitude. Approximately 102,830 km^2 is contained within state boundaries (Woodward and Hoffman 1991). Because of Virginia's variable physiography and climate, the state supports a diverse assemblage of freshwater species. Many boreal species reach the southern limit of their geographic distribution in northern Virginia and many austral species reach the northern limit of their ranges in southern Virginia (Woodward and Hoffman 1991). Additionally, Virginia's major rivers flow in different directions, restricting gene flow between populations and contributing to high species richness. Rivers east of the New River flow southeast to the Atlantic Ocean, the New River flows north to the Ohio River, the Levisa and Russel Rivers of the Big Sandy watershed flow northwest to the Ohio, and other rivers west of the New River flow southwest to the Tennessee River (Fig. 1, Woodward and Hoffman 1991).

Study design

Distributional information was obtained from (1) published literature, (2) unpublished literature authored by R. T. Dillon, (3) museum records, and (4) the Virginia Department of Game and Inland Fisheries. With exception of unattainable publications, we reviewed all peer-reviewed literature that might contain records of freshwater gastropods in Virginia. Information from one unpublished report and a Ph.D. dissertation was also used (Dillon 1977, Dillon 1982). We also included all museum records available on the World Wide Web, specifically those in the Florida Museum of Natural History (FMNH 2002) and the Illinois Natural History Survey Mollusk Collection (INHS 2003). The Virginia Museum of Natural History (VMNH) also provided records. Additional records were obtained from the Virginia Fish and Wildlife Information Service (VDGIF 1998).

A map and/or narrative description of geographic distribution was produced for each species occurring in Virginia now or historically. Records likely resulting from misidentified species were excluded from maps, but are discussed. Species' names and their authorities were based on Turgeon *et al.* (1998).

RESULTS AND DISCUSSION

Our review suggests that 53 species of freshwater gastropods occur in Virginia now or historically. Distributions and ecological requirements for these taxa are discussed below. Our review also uncovered records of eight species that we consider questionable. Records for questionable species are provided with rationale for why we feel they never occurred in Virginia (Table 1).

Family Valvatidae

Valvata tricarinata (Say, 1817). This species reaches the southern extent of its geographic range in Virginia (Clarke

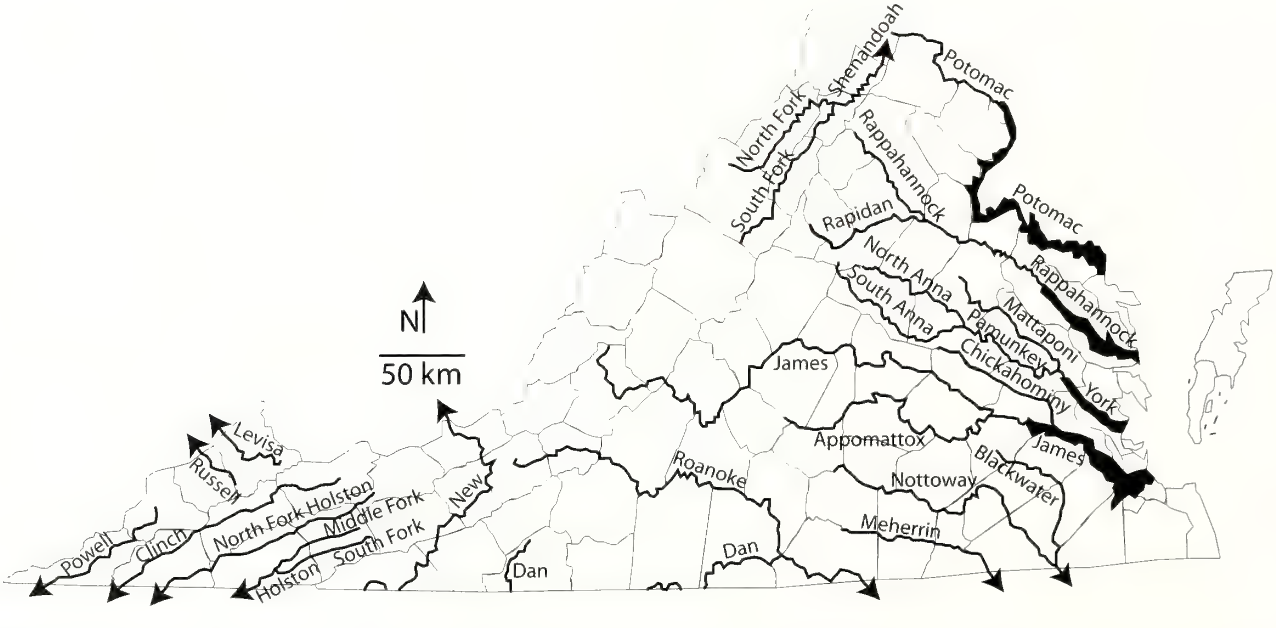


Figure 1. Major rivers of Virginia. Arrows indicate direction of flow (map adapted from Woodward and Hoffman 1991).

Table 1. Although these species were reported from Virginia, it is unlikely that they ever occurred there.

Species	Records (and reference)	Rationale for conclusion
Family Hydrobiidae		
Birgella subglobosus	Fairfax County (Thompson 1977, 1984)	No other records occur near Virginia (Thompson 1977, 1984)
Pyrgulopsis lustrica	Fairfax County (Thompson 1977, 1984)	See *Birgella subglobosus*
Cincinnatia integra	Norfolk (FMNH 2002)	Occurs in the midwestern United States (Burch 1989)
Family Pleuroceridae		
Elimia carinifera	Tazewell County (Beetle 1973a)	Inhabits Alabama River drainage and southern Tennessee River drainage (Burch 1989)
Leptoxis clipeata	Roanoke River, Montgomery County (FMNH 2002)	Now extinct, but occurred in the Coosa River drainage, Alabama (Palmer 1985, Burch 1989)
Lithasia obovata	Augusta and Washington Counties (Burch 1950, FMNH 2002)	Occurs in Ohio River drainage, including states adjacent to Virginia (Burch 1989)
Family Lymnaeidae		
Stagnicola caperata	Fairfax, Page, and Shenandoah Counties (Beetle 1973a)	A northern species that occurs as far south as Maryland (Burch 1989)
Stagnicola oronoensis	Gretna, Pittsylvania County (FMNH 2002)	Inhabits Maine and Ontario (Burch 1989)

1981, Burch 1989, VDGIF 1998). *Valvata tricarinata* is restricted to calcium-rich, permanent, slow-moving waters including lakes and backwaters of large rivers (Baker 1928a, Jokinen 1983, Strayer 1987). Such habitats occur in western Virginia, where this snail has most often been encountered (Woodward and Hoffman 1991, VDGIF 1998). *Valvata tricarinata* has been recorded from Fairfax County, from the Powell River near Back Valley in Lee County, and from the Clinch River near Honaker and Fort Blackmore in Russell and Scott counties (Figs. 1-2, Beetle 1973a, VDGIF 1998).

Family Viviparidae

Viviparus georgianus (Lea, 1834). This is one of four freshwater gastropods to invade Virginia. Presumably due to introductions by aquarists, this native of Florida, Georgia, and Alabama is now discontinuously distributed across the United States (Clench 1962, Clench and Fuller 1965, Mills *et al.* 1993). Populations occupy soft substrates in large, slow-moving bodies of water (Clench and Fuller 1965, Clarke 1981, Strayer 1987). *Viviparus georgianus* has been recorded from the Potomac River near Hunter's Point, from Little Hunting Creek and Mount Vernon in Fairfax County, from Great Creek in Chesterfield County, and from the Wytheville Fish Hatchery in Wytheville, Wythe County (Clench 1962, FMNH 2002).

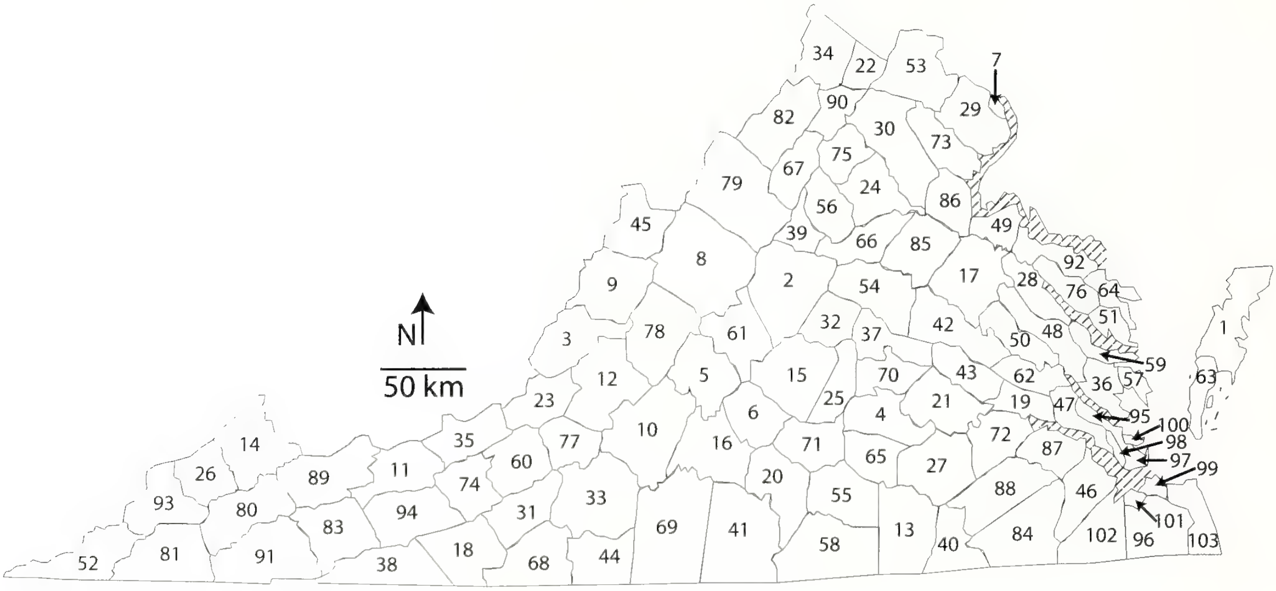
Cipangopaludina chinensis (Reeve, 1863) (= *Viviparus chinensis, Viviparus malleatus* [Reeve, 1863]). This Asian snail has invaded much of North America (Jokinen 1982, Mills *et al.* 1993). It inhabits soft substrata in static or slow-moving waters (Clarke 1981). It is rare in Virginia, but has been reported from the Dyke Marsh in Fairfax County and from the Azalea Gardens in Norfolk (VMNH).

Campeloma decisum (Say, 1817) (= *Campeloma decisa, Campeloma integra* Say, 1821, *Campeloma rufa* [Haldeman, 1841]). Taxonomic confusion surrounds the genus *Campeloma* (Baker, 1928a). Although we found records of *Campeloma crassula* (Rafinesque, 1819) and *Campeloma limum* (Anthony, 1860), these species appear to occur outside of Virginia (Clench and Boss 1967, Dillon 1977, Burch 1989). Thus, we assigned all records of *Campeloma* to *C. decisum*. *Campeloma* has been collected from rivers and streams within the Atlantic, New River, and Tennessee River drainages (Fig. 3A, Goodrich 1913, Burch 1950, Burch 1952, Clench and Boss 1967, Beetle 1973a, Dillon 1977, FMNH 2002, VDGIF 1998, VMNH). This burrowing snail is often abundant in low-gradient rivers and pools with soft substrates (Clench 1962, Clarke 1981, Jokinen 1983).

Lioplax subcarinata (Say, 1816). This species occurs on soft substrates in the Atlantic drainage (Baker 1928a, Clench and Turner 1955, Burch 1989). It has been recorded from Swift Creek in Chesterfield County, the James River near Cartersville in Cumberland County, near Maidens in Powhatan County, Mount Vernon and the Potomac River near Great Falls in Fairfax County, and Fluvanna County (Clench and Turner 1955, Clench and Boss 1967, Beetle 1973a, FMNH 2002).

Family Bithyniidae

Bithynia tentaculata (Linnaeus, 1758) (= *Bulimus tentaculatus*). Most authors feel that North American populations of *B. tentaculata* descended from European snails introduced into the Great Lakes in the 19th century (Baker 1928b, Mills *et al.* 1993). This species is common in the Great Lakes, but uncommon in Virginia where it reaches the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Counties

1. Accomack
2. Albemarle
3. Alleghany
4. Amelia
5. Amherst
6. Appomattox
7. Arlington
8. Augusta
9. Bath
10. Bedford
11. Bland
12. Botetourt
13. Brunswick
14. Buchanan
15. Buckingham
16. Campbell
17. Caroline
18. Carroll
19. Charles City
20. Charlotte
21. Chesterfield
22. Clarke
23. Craig
24. Culpeper
25. Cumberland
26. Dickenson
27. Dinwiddie
28. Essex
29. Fairfax
30. Fauquier
31. Floyd
32. Fluvanna
33. Franklin
34. Frederick
35. Giles
36. Gloucester
37. Goochland
38. Grayson
39. Greene
40. Greensville
41. Halifax
42. Hanover
43. Henrico
44. Henry
45. Highland
46. Isle of Wight
47. James City
48. King and Queen
49. King George
50. King William
51. Lancaster
52. Lee
53. Loudoun
54. Louisa
55. Lunenburg
56. Madison
57. Mathews
58. Mecklenburg
59. Middlesex
60. Montgomery
61. Nelson
62. New Kent
63. Northampton
64. Northumberland
65. Nottoway
66. Orange
67. Page
68. Patrick
69. Pittsylvania
70. Powhatan
71. Prince Edward
72. Prince George
73. Prince William
74. Pulaski
75. Rappahannock
76. Richmond
77. Roanoke
78. Rockbridge
79. Rockingham
80. Russell
81. Scott
82. Shenandoah
83. Smyth
84. Southampton
85. Spotsylvania
86. Stafford
87. Surry
88. Sussex
89. Tazewell
90. Warren
91. Washington
92. Westmoreland
93. Wise
94. Wythe
95. York

Independent Cities

96. Chesapeake
97. Hampton
98. Newport News
99. Norfolk
100. Poquoson
101. Portsmouth
102. Suffolk
103. Virginia Beach

Figure 2. Counties and selected independent cities of Virginia (map adapted from Woodward and Hoffman 1991).

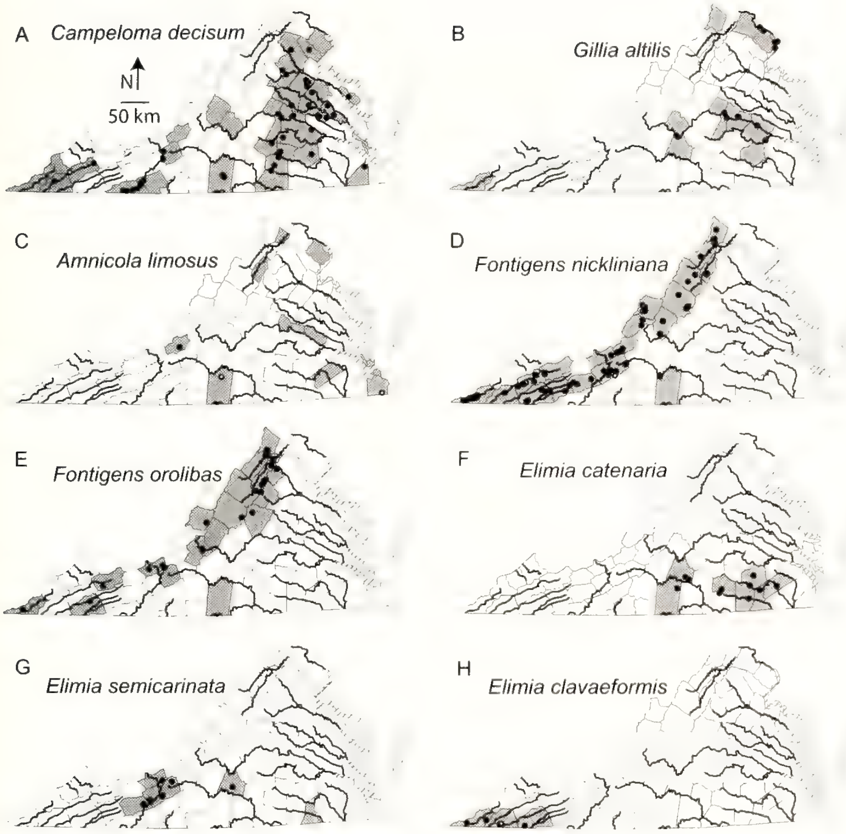

Figure 3. Distributions of (A) *Campeloma decisum,* (B) *Gillia altilis,* (C) *Amnicola limosus,* (D) *Fontigens nickliniana,* (E) *Fontigens orolibas,* (F) *Elimia catenaria,* (G) *Elimia semicarinata,* and (H) *Elimia clavaeformis* in Virginia. Shading indicates counties and independent cities where the taxon has been found. Specific localities of occurrence, if known, are indicated by dots. Filled dots represent records collected during or after 1952. Unfilled circles indicate earlier records. See figures 1-2 for names of rivers, counties, and independent cities.

southern extent of its North American range (Baker 1928a, Burch 1989). It has been recorded from Rockbridge County, and from the Potomac River near Alexandria and Mount Vernon in Fairfax County in the 1920s and 1930s (Pilsbry 1932, Marshall 1933, Beetle 1973a, Dundee 1974).

Family Hydrobiidae

Littoridinops tenuipes (Couper, 1844) (= *Amnicola tenuipes, Bythinella tenuipes*). This species inhabits the Atlantic drainage in streams that are brackish for part of the year (Pilsbry, 1952; Hershler and Thompson, 1992). It has been reported from Hampton, Newport News, and Norfolk cities, and from King George and Northampton counties (Beetle 1973a).

Gillia altilis (Lea, 1841). This species is common in inland rivers and streams (Fig. 3B, Clench and Boss 1967, Beetle 1973a, Thompson 1984, FMNH 2002). Most authors consider *G. altilis* to be restricted to the Atlantic drainage, so Beetle's (1973a) record from Lee County, Virginia (Tennessee drainage) should be examined further (Thompson 1984, Burch 1989).

Somatogyrus virginicus (Walker, 1904). This is one of the most endangered species of freshwater gastropods in North America. The species was described from a population in the Rapidan River at Barnard's Ford, Culpeper County, Virginia (Walker 1904a). Neves (1991) recognized the rarity of records of *S. virginicus* for Virginia, but was unsure if the Rapidan had been sampled after 1904. *Somatogyrus virginicus* has also been reported from North Carolina, but its status there is also uncertain (Neves *et al.* 1997). *Somatogyrus virginicus* is a candidate for federal endangered species status (Neves *et al.* 1997).

Amnicola limosus (Say, 1817) (= *Amnicola limosa*). This species occurs in a variety of habitats of the Atlantic drainage, including lakes, rivers, and permanent streams (Fig. 3C, Rehder 1949, Beetle 1973a, Hershler and Thompson 1988, FMNH 2002, VDGIF 1998).

Lyogyrus granum (Say, 1822) (= *Amnicola grana*). This species is also restricted to the Atlantic drainage, and is often associated with physical structure in eutrophic, slow-moving, permanent waters (Clarke 1981, Burch 1989). It has been reported from Norfolk City, and from Fairfax, Hanover, Henrico, and New Kent counties (Beetle 1973a).

Holsingeria unthankensis (Hershler, 1989). The "thankless ghostsnail" was described as a tiny, pale-colored, stream-dwelling snail inhabiting undersides of stones in Unthanks Cave, Lee County, Virginia (Hershler 1989, Kabat and Hershler 1993). This species is listed as "endangered" by the Virginia Department of Conservation and Recreation, but has not been provided federal protection under the Endangered Species Act (Roble 2001). Before Hershler's (1989) taxonomic revision, *H. unthankensis* and the yet unnamed *Holsingeria* sp. 1 were grouped under the name *Fontigens holsingeri* (Burch 1989, Kabat and Hershler 1993).

Holsingeria sp. 1 *sensu* Hershler (1989). Populations of the "skyline caverns snail" have been collected only from streamside pools in Skyline Caverns, Warren County, Virginia (Hershler 1989, Batie 1991). Although its taxonomic status remains uncertain, this species is certainly imperiled due to limited geographic range (Roble 2001).

Fontigens nickliniana (Lea, 1838). This species and its congeners occur in cool, calcium-rich waters of western Virginia (Hershler *et al.* 1990). It inhabits caves, springs, streams, and small lakes (Fig. 3D, Haldeman 1842, Goodrich 1913, Baker 1928a, Burch 1950, Beetle 1973a, Holsinger and Culver 1988, Hershler *et al.* 1990, Richardson *et al.* 1991, FMNH 2002, VDGIF 1998, VMNH). Hershler *et al.* (1990) list synonyms for this species.

Fontigens orolibas (Hubricht, 1957). This species inhabits springs and cave streams in western Virginia (Fig. 3E, Haldeman 1840-1845, Hubricht 1957, Beetle 1973a, Holsinger and Culver 1988, Hershler *et al.* 1990, Richardson *et al.* 1991, FMNH 2002, VMNH). Hershler *et al.* (1990) list synonyms for this species.

Fontigens morrisoni (Hershler, Holsinger, and Hubricht, 1990). This is another endangered species that is not protected by law, although it is found at only four sites: Blowing and Butler caves in Bath County, and two springs near Mustoe in Highland County (Hubricht 1976, Holsinger and Culver 1988, Hershler *et al.* 1990, Roble 2001). Environmental requirements and life history features are poorly known.

Fontigens bottimeri (Walker, 1925) (= *Paludestrina bottimeri*). This species is limited to a few caves and springs, but has not been granted legal protection (Hershler *et al.* 1990, Roble 2001). In Virginia, it is known only from Ogden's Cave in Frederick County (Hershler *et al.* 1990).

Family Pomatiopsidae

Pomatiopsis cincinnatiensis (Lea, 1840). Limited historic distribution and absence of recent records suggest *P. cincinnatiensis* is imperiled in or extirpated from Virginia. Goodrich (1913) recorded this species from brooks near Cleveland, in Russell County, and Beetle (1973a) reported sightings from Lee and Scott counties. *Pomatiopsis cincinnatiensis* is described as a semiaquatic species (van der Schalie and Dundee 1955). Its habitat consists of a narrow, moist zone on riverbanks (Baker 1928a, van der Schalie and Getz 1962).

Pomatiopsis lapidaria (Say, 1817). This species is also semiaquatic (Baker 1931, Berry 1943). It has more generalized habitat requirements than *Pomatiopsis cincinnatiensis,* and occurs in swampy areas and wet pastures as well as stream edges (Baker 1931, van der Schalie and Dundee 1955). Beetle (1973a) recorded this species from Arlington, Buchanan, Fairfax, Giles, Grayson, Lee, Mecklenburg, Montgomery, New Kent, Patrick, Prince William, Scott, Smyth, Washington, and Wythe counties and from the city of Newport News.

Family Pleuroceridae

Elimia arachnoidea (Anthony, 1854) (= *Goniobasis arachnoidea, Goniobasis spinella* Lea, 1862). This species is limited to small streams in Tennessee and southwestern Virginia (Goodrich 1940, Burch 1989). Goodrich (1913) recorded populations from Little and Big Moccasin Creeks near Gate City in Scott County, and Goodrich (1940) found it in Lee County. Lack of recent records for *E. arachnoidea* makes its present status unclear.

Elimia catenaria (Say, 1822) (= *Goniobasis catenaria*). This species of the Atlantic Coastal Plain has been recorded from several streams and rivers in southcentral Virginia (Fig. 3F, Goodrich 1942, VMNH).

Elimia semicarinata (Say, 1829) (= *Goniobasis semicarinata*). This species occurs in the upper New River and surrounding tributaries and in Campbell and Greensville counties (Fig 3G, Goodrich 1942, Dillon 1977, Dillon and Davis 1980, Dillon 1982, VDGIF 1998). The discontinuous distribution probably indicates an incomplete understanding of its geographic range.

Elimia aterina (Lea 1863) (= *Goniobasis aterina*). This rare species is restricted to a few springs and small streams in Tennessee and southwest Virginia (Goodrich 1913, Burch 1989, FMNH 2002). It has been recorded from Beaver Creek near Bristol in Washington County, and from Stock Creek and a mountain brook near Gate City in Lee County (Goodrich 1913, FMNH 2002). The most recent record was from 1914, so the present status of *E. aterina* is uncertain.

Elimia clavaeformis (Lea, 1841) (= *Goniobasis clavaeformis*). This species is restricted to streams and small rivers in the Tennessee River drainage (Goodrich 1940, Burch 1989). It occurs in tributaries of the Powell and Holston Rivers (Fig. 3H, Goodrich 1913, Beetle 1973a, Dillon 1989, FMNH 2002, VDGIF 1998).

Elimia simplex (Say, 1825) (= *Goniobasis simplex*). This species occurs in the Tennessee and New River drainages (Fig. 4A, Goodrich 1940, Burch 1989). Populations in the New River drainage are restricted to a few small creeks (Fig. 4A, Dillon 1977, Dillon and Davis 1980, Dillon 1982). In the Tennessee drainage, however, *E. simplex* occurs in both small and large rivers, including the Clinch and Holston rivers (Fig. 4A, Say 1825, Tryon 1873, Goodrich 1913, Stansbery and Clench 1974a, 1974b, 1977, Goudreau *et al.* 1993, FMNH 2002, VDGIF 1998, VMNH). This species has also been collected from cave streams (Holsinger 1964, Holsinger and Culver 1988).

Elimia proxima (Say, 1825) (= *Elimia symmetrica* [Haldeman, 1841], *Goniobasis proxima, Goniobasis symmetrica*). This species inhabits Atlantic and Tennessee drainages (Goodrich 1942, Goodrich 1950, Dillon and Keferl 2000). Populations inhabit small streams in addition to large rivers or

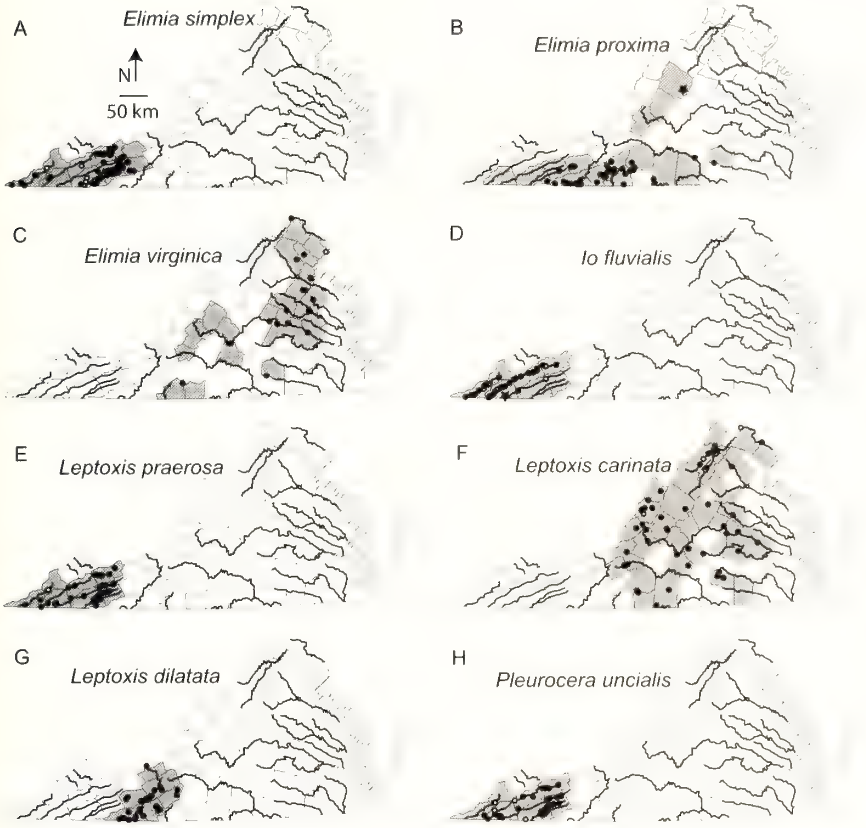

Figure 4. Distributions of (A) *Elimia simplex,* (B) *Elimia proxima,* (C) *Elimia virginica,* (D) *Io fluvialis,* (E) *Leptoxis praerosa,* (F) *Leptoxis carinata,* (G) *Leptoxis dilatata,* and (H) *Pleurocera uncialis* in Virginia. Shading indicates counties and independent cities where the taxon has been found. Specific localities of occurrence, if known, are indicated by dots. Filled dots represent records collected during or after 1952. Unfilled circles indicate earlier records. The star in (B) indicates the location of a population resulting from an introduction by Dillon (1986). The star in (D) indicates the location of a population reestablished as a result of reintroduction efforts (Ahlstedt 1991). See figures 1-2 for names of rivers, counties, and independent cities.

spray zones of falls or springs (Fig. 4B, Tryon 1873, Burch 1950, Goodrich 1950, Dillon 1977, 1982, 1988, Dillon and Davis 1980, Dillon and Keferl 2000, FMNH 2002, VMNH). A population in Coyner Springs, Augusta County, consists entirely of descendents from snails introduced from tributaries of the Dan and New Rivers (Fig. 4B, Dillon 1986).

Elimia virginica (Say, 1817) (= *Goniobasis virginica*). This is the most abundant and widespread species of *Elimia* in large rivers of the Atlantic drainage (Fig. 4C, Burch 1950, Burch 1952, Clench and Boss 1967, Beetle 1973a, FMNH 2002, VDGIF 1998, VMNH). On rare occasions, it is recorded from small streams (Fig. 4C, VMNH). Based on its established range, we concluded that a report of *E. virginica* from Lee County is in error (Goodrich 1942, Burch 1989, FMNH 2002).

Io fluvialis (Say, 1825) (= *Fusus fluvialis, Io brevis* Anthony *in* Reeve, 1860, *Io clinchensis* Adams, 1914, *Io lyttonenesis* Adams, 1914, *Io paulensis* Adams, 1914, *Io powellensis* Adams, 1914, *Io spinosa* Lea, 1837). The "spiny riversnail" occurs in some large tributaries of the Tennessee River, but only in flowing, well oxygenated habitats with abundant limestone (Fig. 4D, Say 1825, Tryon 1873, Adams 1900, Adams 1915, Goodrich 1913, Clench 1928, Lutz 1951, Dazo 1961, Beetle 1973a, Stansbery and Clench 1974a, Stansbery and Stein 1976, McLeod and Moore 1978, Ahlstedt 1979, Ahlstedt 1991, Neves *et al.* 1997, FMNH 2002, INHS 2003, VDGIF 1998, VMNH). Dramatic population declines of *I. fluvialis* from 1900 through the 1970s were documented through field surveys (Adams 1915, Lutz 1951, Stansbery and Stein 1976, McLeod and Moore 1978). Habitat degradation caused by deforestation and industrial pollution destroyed several Virginian populations during this time, including all of those in the North Fork Holston River south of Saltville, Smyth County (Adams 1915, Ahlstedt 1979, Ahlstedt 1991). Due to its small numbers, specific habitat requirements, and continuing threats to its survival, *I. fluvialis* is classified as "threatened" by the Virginia Department of Conservation and has been considered for protection under the federal Endangered Species Act (Neves *et al.* 1997, Roble 2001). Attention directed to *I. fluvialis* has had positive effects. Pollution abatement programs in the 1970s enabled successful reestablishment of *I. fluvialis* in part of its historic range. Ahlstedt (1979) contributed to this recovery in 1978 by reintroducing this species to two sites on North Fork Holston River where it had been absent for almost 100 years. By 1986, Ahlstedt (1991) saw evidence of reproduction and increased population densities at downstream and upstream sites, including one site in Scott County (Fig. 4D).

Leptoxis praerosa (Say, 1821) (= *Anculosa praerosa, Anculosa subglobosa* Say, 1825, *Leptoxis subglobosa, Melania subglobosa*). This species also inhabits tributaries of the Tennessee River (Fig. 4E, Say 1825, Goodrich 1913, 1940, Stansbery 1972, Beetle, 1973a, Stansbery and Clench 1974a, 1974b, 1977, Goudreau *et al.* 1993, Reed-Judkins *et al.* 1998, FMNH 2002, VDGIF 1998, VMNH). *Leptoxis praerosa* is often associated with *Io fluvialis* in large rivers, but *L. praerosa* also occurs in small tributaries that do not support *Io fluvialis* (Figs. 4D, E). Similar to *I. fluvialis, L. praerosa* suffered severe population declines during much of the 20th century (Ahlstedt 1979). However, populations of *L. praerosa* recovered more rapidly than *I. fluvialis* following water quality improvements.

Leptoxis carinata (Bruguière, 1792) (= *Anculosa carinata, Leptoxis nickliniana* Lea, 1839, *Melania nickliniana, Mudalia carinata, Nitrocris carinata, Spirodon carinata*). This is the most abundant and widespread pleurocerid in eastern and central Virginia; densities can reach 500 individuals/m^2 on rocky bottoms of rivers and small creeks (Fig. 4F, Tryon 1873, Pilsbry 1894, Goodrich 1942, Burch 1950, Burch 1989, Clench and Boss 1967, Beetle 1973a, Miller 1985, Dillon 1989, Stewart and Garcia 2002, FMNH 2002, VDGIF 1998, VMNH). *Leptoxis carinata* is restricted to the Atlantic drainage, so we did not plot records from Buchanan, Montgomery, Pulaski, and Wythe counties (Goodrich 1942, Beetle 1973a, FMNH 2002).

Leptoxis dilatata (Conrad, 1835) (= *Nitrocris dilatatus, Spirodon dilatata*). This species occurs in the New River drainage (Fig. 4G, Tryon 1873, Goodrich 1940, Beetle 1973a, Dillon 1977, Burch 1989, Farris *et al.* 1994, Reed-Judkins *et al.* 1998, FMNH 2002). Dillon (1977) found this species to be among the most common molluscs in the upper New River drainage and noted its occurrence in the main river and tributaries. We did not plot records from Alleghany, Amherst, Rockbridge, and Scott counties because these localities are within Atlantic and Tennessee drainages, where *L. dilatata* is replaced by *Leptoxis carinata* and *Leptoxis praerosa,* respectively (Goodrich 1940, Goodrich 1942, Beetle 1973a, FMNH 2002).

Pleurocera canaliculata (Say, 1821) (= *Pleurocera canaliculatum*). This species is restricted to the Tennessee River drainage. Beetle (1973a) reported this species from Lee, Scott, Smyth, Washington, and Wise counties.

Pleurocera gradata (Anthony, 1854) (= *Pleurocera gradatum*). This species has not been seen in Virginia in over 100 years. The only record was from the Holston River, Washington County (Tryon 1873). Despite its rarity, the species has not been granted statewide or federal protection (Roble 2001).

Pleurocera uncialis (Haldeman, 1841) (= *Goniobasis uncialis, Pleurocera "unciale"*). This species occurs in upper tributaries of the Tennessee River (Fig. 4H, Goodrich 1913, 1937, 1940, Beetle 1973a, Stansbery and Clench 1974a, 1974b 1977, Burch 1989, Goudreau *et al.* 1993, Reed-Judkins *et al.* 1998, FMNH 2002, VDGIF 1998). It is the most common species of *Pleurocera* in western Virginia, but pollution has caused declines in abundance (Goudreau *et al.* 1993).

Family Lymnaeidae

Fossaria spp. The taxonomy of the genus *Fossaria* is in a confused state, with species distinguished by minor differences in shell attributes that might be ecophenotypic in origin. Records occurred for the following *Fossaria* "species": *F. humilis* (Say, 1822), *F. dalli* (Baker, 1907), *F. galbana* (Say, 1825), *F. obrussa* (Say, 1825), and *F. parva* (Lea, 1841) (Fig. 5A, Goodrich 1913, Burch 1950, Beetle 1973a, Dillon 1977, Dillon and Benfield 1982, FMNH 2002). *Fossaria* spp. occur in lakes, ponds, and streams, and can thrive in waters with low levels of dissolved oxygen (Baker 1911, Goodrich 1913, Dillon 1977, Dillon 2000). These snails are often semiaquatic, inhabiting moist areas above the water line (Haldeman 1840-1845, Baker 1911, Baker 1928a).

Pseudosuccinea columella (Say, 1817) (= *Lymnaea columella*). This species is found in ponds, lakes, and stream pools across Virginia (Fig. 5B, Rehder 1949, Burch 1950, Burch 1952, Burch and Wood 1955, Beetle 1973a, Dillon 1977, Dillon and Benfield 1982, FMNH 2002). It withstands oxygen fluctuations characteristic of eutrophic habitats, and individuals often occur above the water line on mud and other substrates (Baker 1928a, Jokinen 1983).

Radix auricularia (Linnaeus, 1758). This Eurasian species invaded North America and now occurs at scattered locations (Burch 1989, Mills *et al.* 1993). Populations frequent eutrophic lentic habitats and can be found on mud or plants (Clarke 1979, 1981). In Virginia, *R. auricularia* has only been reported from Giles County (Beetle 1973a).

Stagnicola neopalustris (Baker, 1911). Baker (1911) described a new species of lymnaeid from Orange township, Orange County, Virginia. This is the only known record for this species.

Family Physidae

Physella gyrina (Say, 1821) (= *Physella ancillaria* [Say, 1825], *Physella aurea* [Lea, 1838], *Physella crocata* [Lea, 1864], *Physella elliptica* [Lea, 1831], *Physellaa inflata* [Lea, 1841], *Physella microstoma* [Haldeman, 1840]). Most populations of *Physella* (= *Physa*) show little reproductive isolation (R. T. Dillon, Jr. pers. comm.). Here we synonymize six nominal species reported from Virginia under the oldest veritable name, *P. gyrina*. This species can be found in almost any environment supporting freshwater snails (Clarke 1981, Dillon 2000). However, taxonomic uncertainties and lack of attention directed to pulmonates have resulted in few records for *P. gyrina* or its synonyms (Fig. 5C, Tryon 1865, Walker 1918, Baker 1928a, Burch 1950, Clench and Boss 1967, Beetle 1973a, Wethington *et al.* 2000, VMNH).

Physella acuta (Draparnaud, 1805) (= *Physella hendersoni* [Clench, 1925], *Physella heterostropha* [Say, 1817], *Physella pomilia* Conrad, 1833). After finding no evidence of reproductive isolation among three species of *Physella* (=

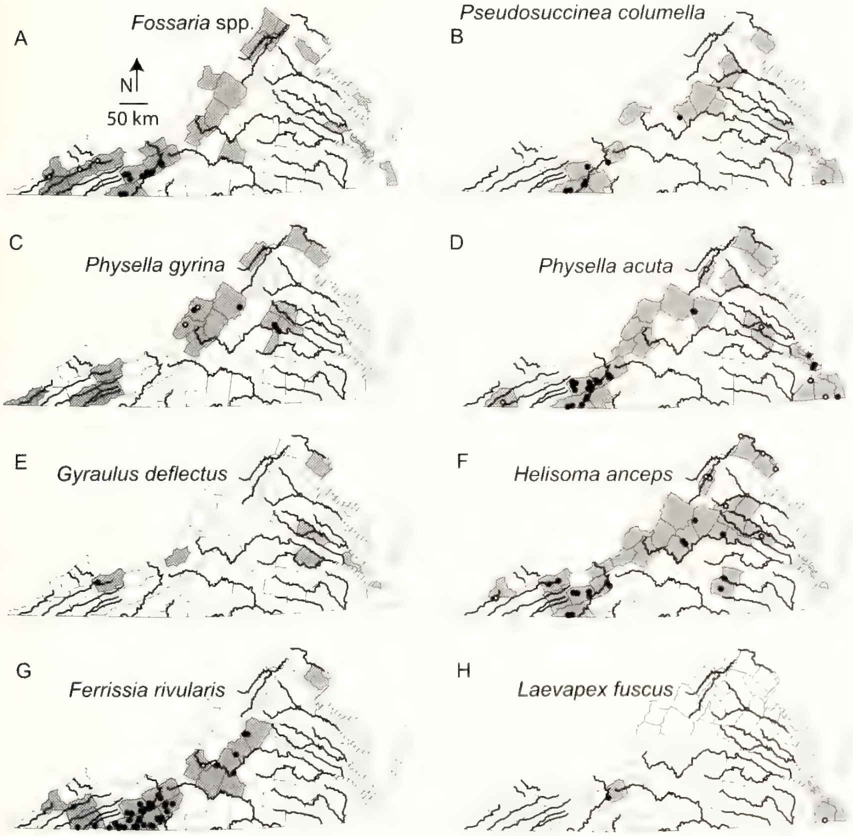

Figure 5. Distributions of (A) *Fossaria* spp., (B) *Pseudosuccinea columella,* (C) *Physella gyrina,* (D) *Physella acuta,* (E) *Gyraulus deflectus,* (F) *Helisoma anceps,* (G) *Ferrissia rivularis,* and (h) *Laevapex fuscus* in Virginia. Shading indicates counties and independent cities where the taxon has been found. Specific localities of occurrence, if known, are indicated by dots. Filled dots represent records collected during or after 1952. Unfilled circles indicate earlier records. See figures 1-2 for names of rivers, counties, and independent cities.

Physa) reported from Virginia, including *P. acuta, P. hendersoni,* and *P. heterostropha,* Dillon *et al.* (2002) assigned the name *P. acuta* to this entire group. *Physella acuta* could be the most abundant and cosmopolitan freshwater gastropod in the world (Dillon *et al.* 2002). The snail occurs in streams, rivers, brooks, ditches, permanent and temporary ponds and lakes and is found on hard and soft substrates (Clarke 1981, Jokinen 1983, Strayer 1987). It inhabits oligo-, meso-, and eutrophic waters (Clarke 1979). Taxonomic confusion and lack of attention directed to pulmonates resulted in few Virginian records for *P. acuta* and its synonyms, although this species probably occurs throughout the entire state (Fig. 5D, Pilsbry 1894, Goodrich 1913, Baker 1928a, Rehder 1949, Burch 1950, Burch 1952, Burch and Wood 1955, Beetle 1973a, 1973b, Dundee 1974, Dillon, 1977, Dillon and Benfield 1982, VDGIF 1998, FMNH 2002).

Aplexa elongata (Say, 1821) (= *Aplexa hypnorum* Linnaeus, 1758). This species is primarily an inhabitant of vernal freshwater habitats, including temporary woodland pools, but has been found in small streams (Baker 1928a, Clarke 1981). It is rare in Virginia (Beetle 1973a, Jokinen 1983, Burch 1989). Records exist from Greene and Surry counties (Beetle 1973a, FMNH 2002).

Family Planorbidae

Gyraulus deflectus (Say, 1824) (= *Gyraulus hirsutus*). This species reaches the southern limits of its geographic range in Virginia and adjacent states (Clarke 1981, Burch 1989). This small snail occurs in mesotrophic and eutrophic lakes, large ponds, and quiet areas of rivers (Fig. 5E, Baker 1928a, Burch 1950, Burch 1952, Burch and Wood 1955, Beetle 1973a, Clarke 1979, Strayer 1987, VDGIF 1998).

Gyraulus parvus (Say, 1817). This species is commonly found in heavily vegetated lakes and ponds, and occasionally lotic habitats (Baker 1928a, Strayer 1987). It has been recorded from Augusta, Frederick, Giles, Rockbridge, Shenandoah, Wythe, and York counties and the city of Newport News (Burch 1950, Beetle 1973a).

Helisoma anceps (Menke, 1830) (= *Helisoma antrosa* Conrad, 1834, *Planorbis bicarinatus* Say, 1819). This species is common throughout Virginia (Fig. 5F, Pilsbry 1894, Walker 1909, Goodrich 1913, Baker 1945, Burch, 1950, Burch 1952, Burch and Wood 1955, Clench and Boss 1967, Beetle 1973a, Dillon 1977, Dillon and Benfield 1982, Burch 1989, FMNH 2002, VDGIF 1998, VMNH). Among planorbids, this species is unusual in that it is most commonly found in lotic habitats, although it also inhabits ponds and lakes (Jokinen 1983, Dillon 2000).

Micromenetus brogniartianus (Lea, 1842) (= *Menetus brogniartianus*). Both global and Virginian distributions of this small planorbid are poorly known (Burch 1989). It was reported from Surrey County and the city of Newport News (Beetle 1973a).

Micromenetus dilatatus (Gould, 1841) (= *Menetus dilatatus*). Baker (1945) recorded this species from the vicinity of Luray, Page County, Virginia. Additional records exist from Culpeper, Fairfax, New Kent, and Prince William

Counties and the cities of Hampton and Newport News (Beetle, 1973a). This species is encountered in vegetated lentic habitats and also in upland streams (Jokinen 1983, Strayer 1987).

Planorbella trivolvis (Say, 1817) (= *Helisoma trivolvis*). Although this large planorbid is distributed throughout the eastern and midwestern United States, we found few records (Baker 1928a, Burch 1989). It has been reported from the Holston River, near Marion in Smyth County, and from Fairfax County and the cities of Newport News and Norfolk (Beetle 1973a). *Planorbella trivolvis* occurs in lentic habitats and areas of slow flow in rivers and streams (Baker 1928a, Clarke 1981).

Planorbella armigera (Say, 1821). This snail is usually associated with vegetation in perennial, lentic habitats (Baker 1928a, Clarke 1981, Burch 1989). It has been reported from Fairfax County and the cities of Hampton, Newport News, and Virginia Beach (Beetle 1973a).

Promenetus exacuous (Say, 1821) (= *Menetus exacuous*). This species inhabits still areas of permanent and vernal freshwater habitats (Baker 1928a, Clarke 1979, Burch 1989). It has been recorded in Dinwiddie and Prince George counties (Beetle 1973a).

Family Ancylidae

Ferrissia fragilis (Tryon, 1863) (= *Ancylus pumilus* Lea, 1845, *Ferrissia californica* [Rowell, 1863], *Ferrissia shimekii* [Pilsbry, 1890], *Gundlachia meekiana* Stimpson, 1863). This tiny gastropod occurs in lentic, eutrophic waters (Basch 1963, Clarke 1979, Burch 1989). Walker (1904b) recorded this species from Fairfax County near Alexandria, Virginia. Additional records occur from Hanover and Rockbridge counties (Burch 1952, Beetle 1973a).

Ferrissia parallela (Haldeman, 1841) (= *Ancylus parallelus*). This limpet reaches the southern extent of its range in northern Virginia (Basch 1963, Burch 1989). The only record we found was from Fairfax County (Beetle 1973a). *Ferrissia parallelus* inhabits lentic habitats where it is often found clinging to vegetation (Baker 1928a, Clarke 1981).

Ferrissia rivularis (Say, 1817) (= *Ancylus depressus* Haldeman, 1844, *Ancylus haldemani* Bourguignat, 1853, *Ancylus rivularis, Ancylus tardus* Say, 1830). This is the most commonly reported ancylid in Virginia (Fig. 5G, Haldeman 1840-1845, Walker 1904b, Basch 1963, Beetle 1973a, Dillon 1977, Reed-Judkins *et al.* 1998, VDGIF 1998). *Ferrissia rivularis* lives in lotic habitats, where it occupies cobbles or other hard substrates in riffles (Baker 1928a, Jokinen 1983).

Laevapex fuscus (Adams, 1841) (= *Ancylus fuscus, Ferrissia fusca*). This species typically inhabits lakes and slow-flowing areas of streams and rivers, with occasional records of collections from rivulets (Fig. 5H, Rehder 1949, Beetle 1973a, Dillon 1977). *Laevapex fuscus* can be found attached to vegetation, rocks, and man-made objects (Baker 1928a, Basch 1963).

CONCLUSIONS

A diverse group of freshwater gastropods inhabits Virginia, with more than 50 species occurring there historically or presently. However, the lack of recent records for several species is cause for concern, as is evidence that some species are found only at one or two locations. Because intensive survey efforts have been directed to them, it is clear that *Fontigens bottimeri, Fontigens morrisoni, Holsingeria unthankensis,* and *Holsingeria* sp. 1 are extremely rare and endangered. Other species with few records, including *Somatogyrus virginicus, Pomatiopsis cincinnatiensis, Elimia arachnoidea, Pleurocera gradata,* and *Stagnicola neopalustris* are also likely endangered or extirpated. However, field surveys are still needed to determine their statuses.

Other taxa and specific geographic regions should also be surveyed. Specifically, pulmonates (families Lymnaeidae, Physidae, Planorbidae, and Ancylidae) have been undersurveyed, thus their maps underestimate their distributions. Additionally, we found no records from the Big Sandy drainage, including Buchanan and Dickenson counties and the Levisa and Russel Rivers, and only one record (*Littoridinops tenuipes*; Beetle 1973a) from the eastern shore (i. e., Accomack and Northampton Counties). Absence of records from these regions are in contrast to the Tennessee drainage in southwest Virginia, where detailed surveys of rivers revealed declines and subsequent recoveries of pleurocerid populations (Adams 1915, Ahlstedt 1979, 1991).

By summarizing survey data from different sources, we hope to stimulate research that will improve our understanding of the freshwater gastropod fauna of Virginia. We identified species and geographic regions that have been well surveyed, as well as those requiring additional study. Furthermore, we provide critical baseline data for measuring temporal changes in gastropod abundance and distributions. Comparisons of data from historic and future field surveys will facilitate legal protection of endangered species by providing evidence of restricted or shrinking geographic ranges. Consequently, effective management plans can be developed for species in need of assistance.

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Environmentally and genetically induced shell-shape variation in the freshwater pond snail *Physa* (*Physella*) *virgata* (Gould, 1855)*

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Abstract: Species of North American freshwater snails within the genus *Physa* are distinguished primarily on differences in shell shape. However, shell shape in this genus is ecophenotypically influenced by environmental factors. The present study examined the degree to which genes and environment influenced spire angle in specimens of *Physa virgata* collected from a single population in Arlington, Texas. Five separate genetic lines were inbred for five generations, after which progeny were reared under one of three temperature regimes (20°, 25°, or 30°C) until reaching adult size (approximately 5 mm in shell length). The spire angle of each individual was then measured and comparisons made across thermal regimes and genetic lines. Genetic line and temperature both significantly influenced spire angle. In general, snails reared at warmer temperatures had wider spire angles than snails reared at cooler temperatures. Some genetic lines had wider mean spire angles than others, regardless of temperature. Individual spire angles differed by as much as 23.8° under controlled conditions. These results suggest that shell shape is neither a consistent character nor taxonomically diagnostic. Thus, the number of currently recognized species of *Physa* is problematic.

Key words: *Physa*, shell shape, reaction norms, phenotypic plasticity

Species with populations occupying heterogeneous environments are exposed to different, often dynamic, environmental conditions. Thus, different populations of a given species may be acted upon by differing selective pressures, resulting in no exclusively optimal phenotype for the species in general (Via and Lande 1985). In response to naturally varying selective pressures, many such species have evolved the ability to modify an individual's phenotype in response to environmental cues (Pigliucci 2001). This phenotypic plasticity may potentially allow populations to inhabit otherwise hostile environments by providing protection from local hazards (Via and Lande 1985). However, the ability to modify phenotype is almost certainly constrained by costs such as those associated with sensory and regulatory mechanisms related to plasticity, and limitations to benefits such as those imposed by unreliable environmental cues (reviewed by DeWitt *et al.* 1998).

Life history patterns, in particular, are well studied in relation to environmental modification. For example, intraspecific competition affects growth rates in freshwater pulmonates. Brown (1979) demonstrated that increasing population densities of experimental populations of the freshwater snail *Lymnaea stagnalis* Linnaeus, 1758 led to reduced growth rates and delayed maturity in this species. Similarly, individuals of *Physa gyrina* (Say, 1821) decreased shell growth rates as population density increased (Brown, 1982). Yet, growth rate in this species increased and fecundity decreased when individuals of *P. gyrina* were reared in competition with *Stagnicola elodes* (Say, 1821) (Brown 1982). Kawata and Ishigami (1992) similarly reported that individuals of *Physa acuta* (Draparnaud, 1805) exposed to water containing chemical cues from of a second (naturally sympatric) species (*Lymnaea* sp.) exhibited accelerated growth rates relative to individuals exposed to water conditioned by conspecifics and controls (without snail cues).

The presence or absence of predators has also been demonstrated to affect phenotypic characters (Crowl 1990, Crowl and Covich 1990, DeWitt 1996). Crowl and Covich (1990) reported that when exposed to chemical cues created by crayfish feeding on conspecifics, experimental populations of *Physa* (*Physella*) *virgata* (Gould, 1855) accelerated growth rates and decreased reproductive rates until reaching a larger size relative to populations not exposed to the cues. Crowl (1990) reported that the presence of predators (crayfish) is at least as important as environmental instability (measured by stream permanence) in inducing life-history trait variation in *P. virgata*.

Life-history traits, however, are not the only phenotypic characters in freshwater gastropods influenced by environmental cues. DeWitt (1996) showed that individuals of *Physa (Physella) heterostropha* (Say, 1817) exposed to a fish predator produced rounder shells with slower growth rates compared to snails exposed to a crayfish predator, which produced more elongate shells with faster growth rates. Burnside (1998) demonstrated that population density and experimentally manipulated growth rates were also associated with differential shell shapes in *P. virgata*, with higher densities and faster growth rates leading to rounder apertures.

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Phenotypic plasticity in shell shape is under-appreciated and problematic with regard to freshwater pulmonate snails because many species are distinguished based on shell shape parameters. If morphological variation can be induced by environmental cues, then the validity of routine use of shell shape parameters as indicators for gastropod species identification must be questioned.

The freshwater snail genus *Physa* Draparnaud, 1801 (Pulmonata: Basommatophora) is a worthy choice for illustrating how shell shape parameters might be problematic for the identification of species of freshwater pulmonates. Currently, physid species are primarily or solely based on slight variations in shell shape (Te 1975, Burch 1989). Although anatomical differences exist between some currently accepted species, they are often relatively minor, with several accepted species sharing similar anatomy (Te 1973, 1975). Te (1973) reported that species of *Physa* include only a handful of complexes that can be distinguished based on differences in penile anatomy. Nevertheless, approximately 40 species of *Physa* are currently recognized (Turgeon *et al.* 1998), and are typically identified by comparing collected specimens to Burch's (1982, 1989) illustrations of physid shells, which have little or no description of anatomical or other physical differences.

Many gastropods, including physids, are known to have considerably plastic shell shapes influenced by both biotic and abiotic factors, including water velocity (Urabe 1998), presence or absence of predators (Appleton and Palmer 1988, DeWitt 1996, 1998), population density and growth rate (Burnside 1998). If characteristics of shell shape are assumed to be consistent within a species but are actually ecophenotypically plastic, then measured differences between geographically distinct populations may lead to erroneous species designations if shell shape is the primary or sole characteristic considered. Thus, more species of *Physa* could be described than actually exist. Many of the currently identified North American physid species may be ecophenotypic variants of a much smaller number of reproductively distinct species. Carefully planned breeding experiments are one way to test whether species are distinct. Dillon *et al.* (2002) were able to synonymize *Physa integra* (Haldeman, 1871) and *Physa heterostropha* with *Physa acuta*, showing a clear lack of reproductive isolation between these phenotypically similar "species."

Consistency of any character, including shell shape, within a species is essential for it to be taxonomically useful. Because both genetic and environmental conditions are likely to affect shell shape, it is important to examine the relative degree to which each exhibits influence. The objective of this study was to investigate the degree to which the shape of the shell of *Physa virgata* can be influenced by controlled genetic and environmental conditions and to address the validity of shell shape as a taxonomically useful character for this genus.

MATERIALS AND METHODS

Thirty immature juveniles of *Physa virgata* less than 4.0 mm in shell length (SL) were collected from a single population in Trader Horse Creek on the University of Texas at Arlington Campus, Arlington (Tarrant County), Texas, USA (32.72739°N, 97.11274°W). Sampled snails were returned to the laboratory and isolated in 250-ml plastic containers filled with de-chlorinated tap water, maintained in an incubator at 25°C under a 12-hr light/dark cycle. Snails were fed Wardley® Total Tropical® flake fish food *ad libitum*. Each isolated individual was used to produce a separate, random genetic line of progeny by allowing it to mature in isolation and reproduce by self-fertilization, creating a genetic bottleneck for its subsequent line of progeny.

Offspring were left in parental containers until they reached about 0.5 mm in shell length. At this time, the parent and all but three or four juveniles were removed. The remaining juveniles were maintained in the same manner as their parent, except that they were allowed to inbreed with siblings or self-fertilize after reaching sexual maturity. No attempt was made to determine if individual snails self-fertilized or outcrossed with siblings. A total of five generations of progeny were produced in the laboratory in this manner. Because individuals of *Physa virgata* are diploid, a heterozygote for a hypothetical locus (Aa for example) could potentially produce offspring with three different genotypes (AA, Aa, or aa) even if the individual reproduced by self fertilization. However, restricting reproduction to selfing or outcrossing among siblings prevented new alleles from adding genetic variation to each line. Additionally, inbreeding within such small laboratory populations could potentially remove rare alleles over generations by way of genetic drift. Thus, inbreeding allowed maintenance or reduction of genetic variation within each genetic line over each generation.

Growth and reproductive rates were highly variable among the 30 lines. Some lines produced mostly fast-growing progeny while others produced only a few, non-viable, or slowly growing offspring. Thus, 5 of the original 30 genetic lines were chosen for all subsequent experimentation based on their ability to consistently produce numerous, viable offspring.

After five generations of inbreeding at 25°C, approximately 15 hatchlings (<0.5 mm SL) were collected from each genetic line. Each selected hatchling was isolated in a new 250-ml plastic container and randomly assigned to a thermal regime of 20°, 25°, or 30°C. Individual genetic lines did not produce equal numbers of viable offspring. Each of the three thermal regimes received as many snails as were available. A minimum of five snails (range = 5 to 12 snails) from each of the five genetic lines were assigned to each treatment. It was hypothesized that different thermal regimes would induce different growth rates and thereby indirectly influence shell shape. Snails remained in containers within incubators under a 12-hr light/dark cycle and were fed *ad libitum*. When each F2 snail approached about 5 mm in SL (range approximately 3 to 7 mm), it was removed and stored in 70% ethanol. Snails that perished during the experiment were discarded. Shell growth rates were estimated by subtracting 0.5 mm (i.e., the approximate hatchling shell length when initially isolated) from the final shell length and dividing by the number of weeks elapsed under treatment.

Digital images of all snail shells were captured with shells held in a position corresponding to the images of Burch (1989). Images were captured with a Sony CCD-IRIS color video camera (Model DXC-107A) fitted with a Navitar 7000 TV zoom lens. The long axis (from apex of the spire to the most distant point along the edge of the aperture) and apertural plane were held parallel to the focal plane of the camera. Morphometric measurements were recorded using digital image analysis software (SigmaScan Pro 5.0 by SPSS), including shell length in mm and spire angle in degrees. Shell length was measured along the long axis as described above, and spire angle was measured from the penultimate whorl to the apex of the spire (Fig. 1).

Because allometric shell growth is common in gastropods, it was necessary to assess whether shell length and/or growth rate should be included as covariates when testing whether spire angles differed between treatments. As a rough evaluation of whether shell length was the major factor influencing spire angle (i.e., allometric growth had occurred), a reduced major axis (RMA) regression of shell length (independent variable) on spire angle (dependent variable) was performed using data combined from all genetic lines. Five additional independent RMA regressions were also performed, each examining the relationship between shell length and spire angle for a specific genetic line.

Similarly, an RMA regression was performed to test whether growth rate (independent variable) influenced spire angle (dependent variable) using data combined for all genetic lines. The relationship between growth rate and spire angle was also evaluated independently for each genetic line resulting in five additional RMA regressions. Type II (RMA) regressions were necessary because the independent variables (SL and Growth Rate) were not measured without error, an assumption in type I regression.

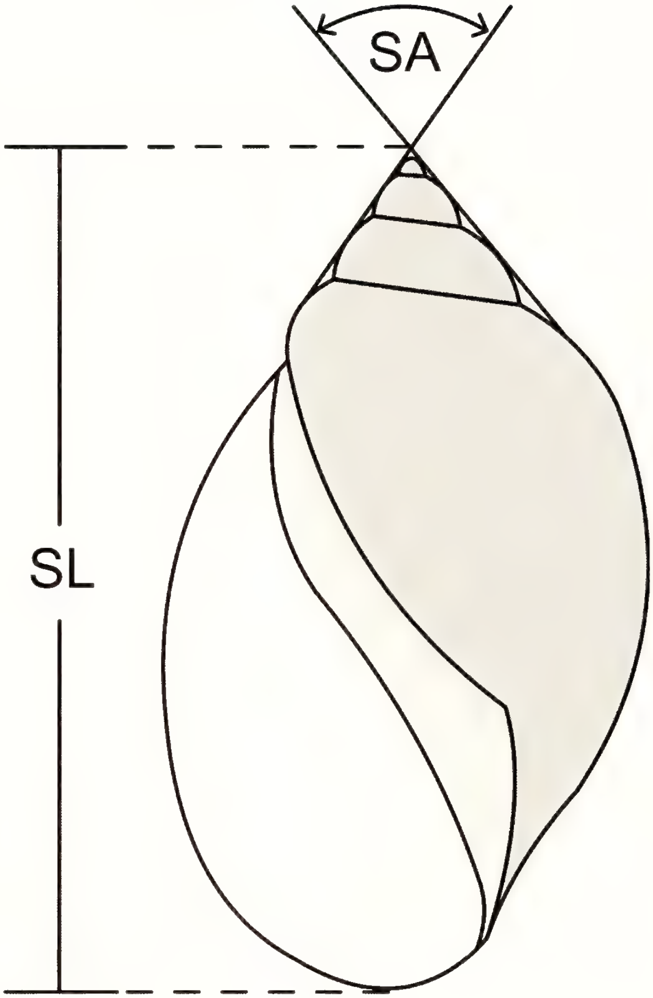

Figure 1. Shell measurements made on specimens of *Physa virgata*. SA is spire angle measured in degrees. SL is shell length measured in mm.

Mixed-model two-way ANCOVAs were performed to assess whether growth rates and spire angles differed among treatments. Shell length was included as covariate in the first ANCOVA (assessing growth rate means across treatments) and shell length and growth rate were included as covariates in the second (assessing spire angle means across treatments). Genetic line (arbitrarily assigned 1, 2, 3, 4, or 5) and thermal regime (20°, 25°, or 30°C) were independent variables (with genetic line treated as a random variable) in both ANCOVAs.

RESULTS

Growth rates for individuals of *Physa virgata* ranged from 0.049 to 0.405 mm/day, averaging 0.146 mm/day (sd =

0.077) with data combined across all genetic lines. Shell spire angles ranged from 55.2° to 79.0° with a mean of 66.6° (sd = 5.5), including data from all lines (Fig. 2). Measurements were made on snails ranging in shell length (SL) from 3.0 to 6.9 mm with a mean of 4.9 mm (sd = 0.80).

The reduced major axis (RMA) regression of shell length on spire angle that combined data for all genetic lines and thermal regimes, although statistically significant at $\alpha = 0.05$ ($r^2 = 0.046$, $p = 0.043$), suggested that shell length was not a major factor influencing spire angle over the range of shell sizes studied (Fig. 3). Two of five independent RMA regressions of shell length on spire angle for individual genetic lines were statistically significant (Genetic Line 4, $r^2 = 0.273$, $p = 0.01$; Genetic Line 5, $r^2 = 0.198$, $p = 0.03$) with both indicating a negative correlation between shell length and spire angle. Neither of these accounted for more than 27.3% of the variation revealed in spire angle (Fig. 3). The three independent RMA regressions for the remaining genetic lines did not reveal a significant ($\alpha = 0.05$) relationship between shell length and spire angle.

Although growth rates varied substantially in this study, they did not significantly influence shell shape measured as spire angle. The RMA regression of growth rate on spire angle, combining data from all genetic lines and thermal regimes, was not significant ($r^2 = 0.0014$, $p = 0.73$). Moreover, none of the individual RMA regressions of growth rate on spire angle for individual genetic lines were significant (Fig. 4).

ANCOVA revealed a significant effect of inbred line as well as an interaction between the effects of temperature and inbred line on growth rate (Table 1). The influence of temperature on growth rate was dependent on genetic line (Fig. 5), although some lines had consistently higher growth rates than others, regardless of temperature.

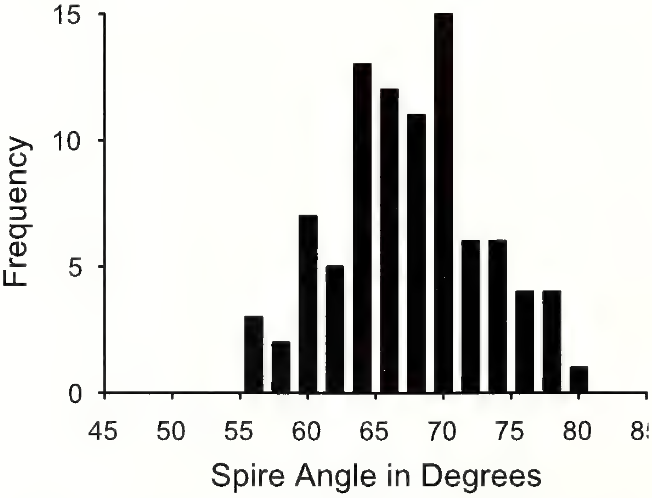

Figure 2. Histogram of the distribution of spire angles measured from all specimens of *Physa virgata* utilized in experiments.

Both genetic line and temperature significantly influenced spire angle (Table 2). Some genetic lines had wider spire angles than others regardless of temperature, and the snails in the 25° and 30°C regimes had wider spire angles than those in the 20°C regime (Fig. 6). The relationship between temperature and spire angle was fairly consistent and not confounded by an interaction with inbred line. By dividing the variation for each effect by the sum of the measured variation (sum of squares) for all effects (temperature, genetic line, and interaction) one can estimate the relative proportion that each effect contributed to the total measured variation in spire angle. Genetic line, temperature regime, and interaction accounted for approximately 44%, 37%, and 19%, respectively, of the measured variation in spire angle in individuals of *Physa virgata*.

DISCUSSION

When data from all specific genetic lines were combined, the total sample reflected a reasonably normal distribution of spire angles with a mean of approximately 67°. In comparison, Burch's (1989) illustration of *Physa virgata* had a spire angle of approximately 73°. The standard deviation of the studied sample was 5.5°, making the shape of Burch's (1989) illustrated specimen consistent with the expected distribution based on spire angles obtained in the present study. Indeed, assuming that spire angles are normally distributed and there is nothing special about the Trader Horse Creek population of *P. virgata*, one should expect 95% of adult individuals of *P. virgata* to have a spire angle somewhere between 55.6° and 77.6° (two standard deviations on either side of the mean). Remarkably, this range includes the spire angles of most of the physid species depicted in Burch (1989).

Although it was anticipated that shell growth rate would influence shell shape in *Physa virgata*, this was not demonstrated. The relationship between shell growth rate and temperature was dependent on genetic line examined. Specifically, temperature had little impact on the shell growth rates of Lines 1 and 5 (a change of about 0.04 mm/day from 20°-30°C), but had substantial influence on Line 2 (an increase of >0.25 mm/day over the same temperature range). Two of the three lines had maximal growth rates at 25°C while three had maximal rates at 30°C (Fig. 5). Although growth rate did not influence adult spire angle in this study as expected, temperature did. In general, higher temperatures (25° and 30°C) corresponded to wider spire angles in *P. virgata*. The relationship between spire angle and temperature produced generally similar reaction-norm patterns for

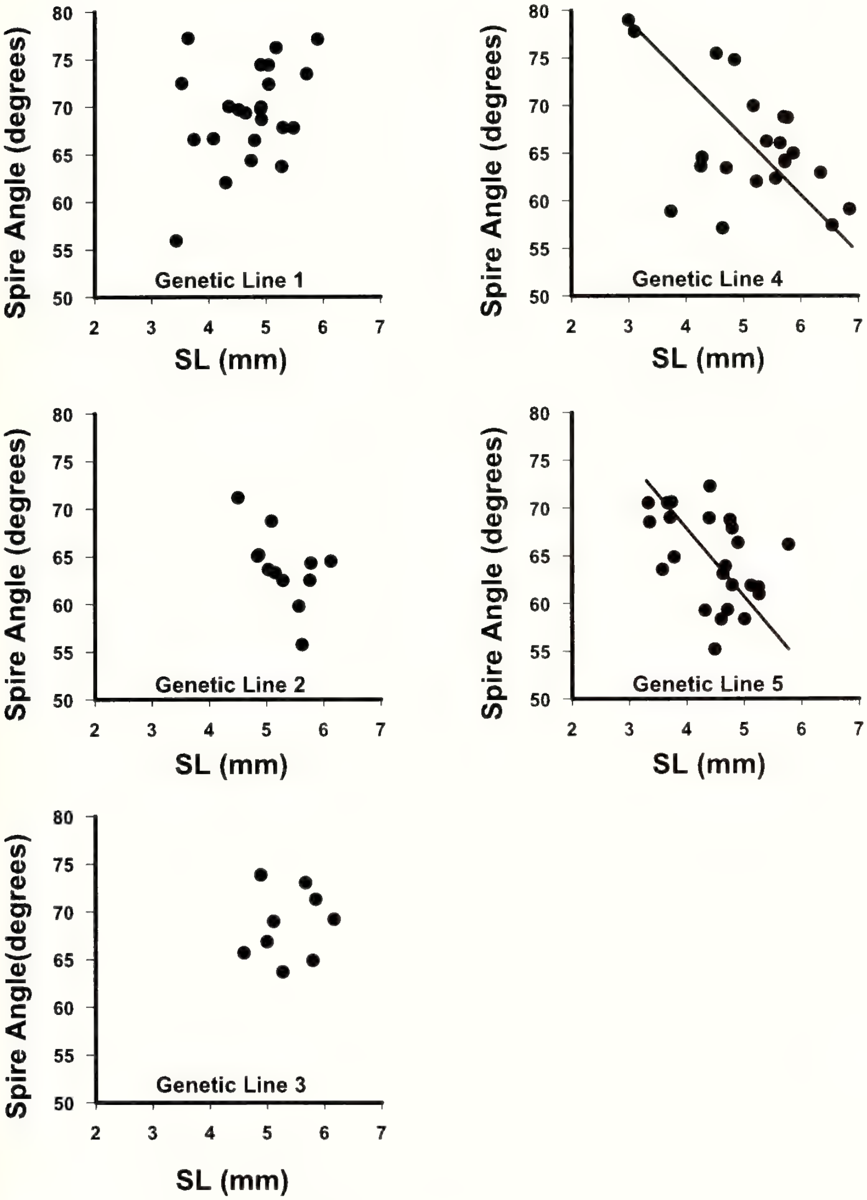

Figure 3. Scatter plots of spire angle vs. shell length in specimens of *Physa virgata*. Data from each genetic line are indicated in separate panels. Shell length was not significantly correlated with spire angle when all data were combined. The relationship was also not significant for three genetic lines when examined separately. However, two genetic lines had significant regressions: both Genetic Line 4 ($r^2 = 0.273$, $p = 0.01$) and Genetic Line 5 ($r^2 = 0.198$, $p = 0.03$) indicated a negative correlation (depicted in the figure as solid lines) between shell length and spire angle but explained very little of the observed variation.

each genetic line, although each was vertically shifted with respect to the others, indicating a genetic influence on spire angle (Fig. 6). The slopes of these reaction norms can be attributed to the ecophenotypic influence of the environmental variable (temperature), and the height of the lines can be attributed to the genotypic influence (Pigliucci 2001).

The individuals of *Physa virgata* utilized in this study were all derived from a single population taken from one location during a single collection event. Nevertheless, shell shape and shell growth rates varied substantially among individuals. Although most measured variation in spire angle was attributable to genetic line (44%), a considerable proportion (37%) was attributable to environmentally induced differences (i.e., temperature regime). After isolating distinct genetic lines and controlling rearing temperature, snails produced descendants with remarkably different shell shapes. Spire angles ranged from approximately 52° to 79°, as much or more variation in spire angle than depicted in the illustrations of the shells of the over 40 identified North American species of *Physa* (Burch 1982, 1989).

Physa virgata is an *r*-selected invasive species, as are most freshwater pulmonates (McMahon 1983). It has evolved tolerance of the varying conditions typical of the small, ephemeral ponds and streams where this species is often found (McMahon 1983). The ability to self-fertilize, capacity to produce numerous offspring, and ability to readily disperse (aided, perhaps, by transport on the feet of waterfowl), allows single individuals to quickly found new populations and/or repopulate ephemeral ponds and streams when water returns (Dillon and Wethington 1995, Dillon 2000). This study suggests that individuals (including those that found new populations) can be genetically predisposed for wider (or narrower) spires, and that this genetic predisposition can be ecophenotypically enhanced or diminished by non-genetic, environmental influences such as temperature. Although the extensive genetic variation in shell shape revealed in this study could be the basis for the evolution of adaptive shell shapes within populations subjected to long-term, specific selection pressures (e.g. crayfish predation or rapid water velocity), a large proportion of variation in the spire angle of

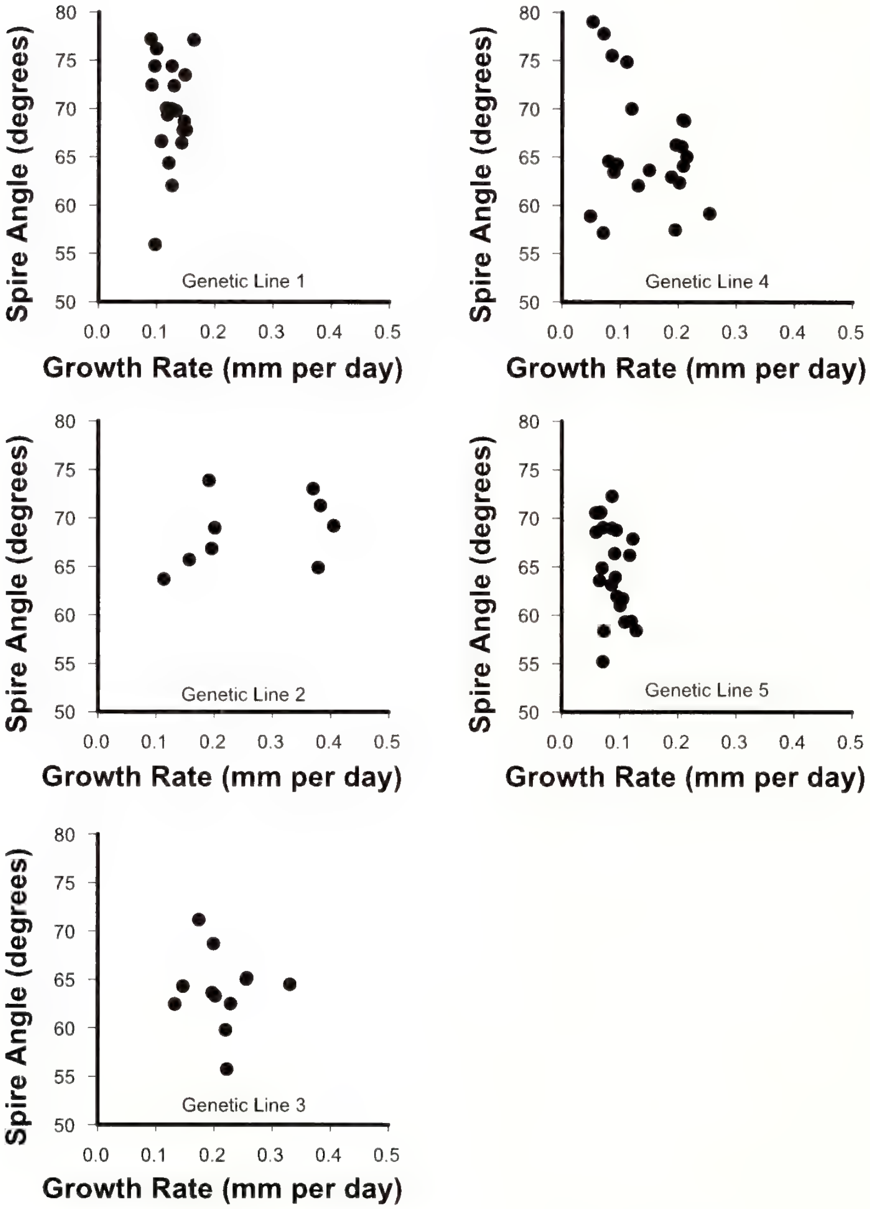

Figure 4. Scatter plots of spire angle vs. growth rate in specimens of *Physa virgata*. Data from each genetic line are indicated in separate panels. Growth rate was not significantly correlated with spire angle when data were combined for all genetic lines or when data for each genetic line were examined separately.

measured individuals of *Physa virgata* was estimated to be environmentally induced. Thus, it is possible that non-genetic, ecophenotypic influences on a single North American species of *Physa* could generate much of the variation in shell shape presently attributed to the many different, and potentially problematic, North American species recognized in this genus.

The adaptive significance of wider spire angles in association with increased temperatures is unknown. However, DeWitt (1996) successfully demonstrated similar phenotypic shell-shape plasticity in individuals of *Physa heterostropha* and reported narrower apertures in snails exposed to crayfish predators and rounder apertures in snails exposed to fish predators. DeWitt (1996) argued that rounder apertures offer more structural stability and protection for snails exposed to shell-crushing predators like molluscivorous sunfish while snails with elongate shells and narrow apertures could better deter shell-entry predators such as crayfish. Thus, phenotypic plasticity likely offers considerable advantages to individuals of this wide-spread genus known to occupy extensively unpredictable heterogeneous habitats with varying hazards. Interested readers are encouraged to review the discussions of the implications of phenotypic plasticity in general in the context of ecology discussed in Pigliucci (2001) and DeWitt *et al.* (1998).

Recent evidence demonstrated that North American species of *Physa* (i.e., *Physa integra*, *Physa heterostropha*, and *Physa acuta*) are capable of interbreeding without reduction in fecundity in hybrids (Dillon *et al.* 2002). The present study provides additional support for the suggestion that many presently recognized North American species of the subgenus *Physella* Haldeman, 1842 may represent ecophenotypes of a single or relatively few wide-spread species with (highly) environmentally plastic shell shapes (Dillon *et al.* 2002).

Spire angle is only one of many characteristics of shell shape that are frequently used to distinguish freshwater gastropod species. However, many shell shape metrics are often correlated. Because considerable variation exists in spire angle within this species, it is likely that substantial variation also occurs in other characteristics of the shell shape. DeWitt (1996) has also demonstrated ecophenotypic variation in aperture roundness in *Physa heterostropha.* Thus, until shell shape characters can be shown to be consistent within a gastropod species, they should not be utilized as the primary diagnostic characters for species identification.

Table 1. ANCOVA: Temperature and Inbred Line on Growth Rate with Shell Length treated as a covariate.

Effect	*df*	MS Effect	MS Error	*F*	*p*
Genetic line	4, 73	0.2148	0.00065	32.9	<0.0001**
Temperature	2, 8	0.0192	0.01009	1.90	0.211
Interaction	8, 73	0.0101	0.00065	15.4	<0.0001**

** $p << \alpha = 0.05$

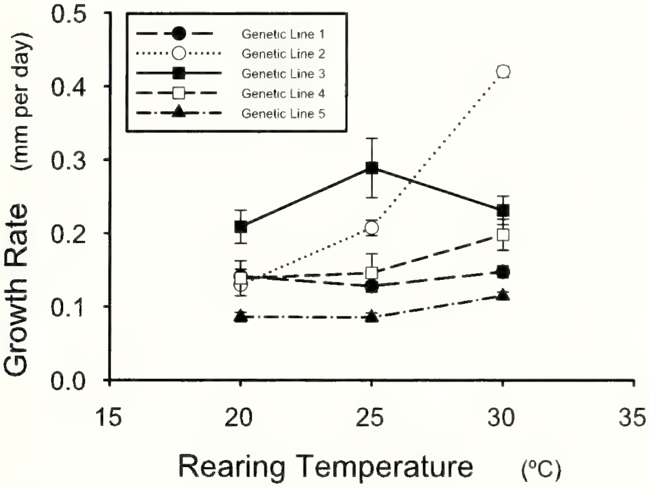

Figure 5. Mean shell growth rate vs. rearing temperature for specimens of *Physa virgata*. Shell growth of different genetic lines (distinguished by symbols) responded differently to increases in rearing temperature. The slopes of lines connecting means represent environmental effects on growth rate while the relative height of the lines represents genetic effects on growth rate. Error bars represent one standard error of the mean.

Table 2. ANCOVA: Temperature and Inbred Line on Spire Angle with Shell Length and Growth Rate treated as covariates.

Effect	*df*	MS Effect	MS Error	*F*	*p*
Genetic Line	4, 72	82.05	19.55	4.19	0.004*
Temperature	2, 8	137.22	18.17	7.55	0.014*
Interaction	8, 72	18.17	19.55	0.929	0.498

* $p < \alpha = 0.05$

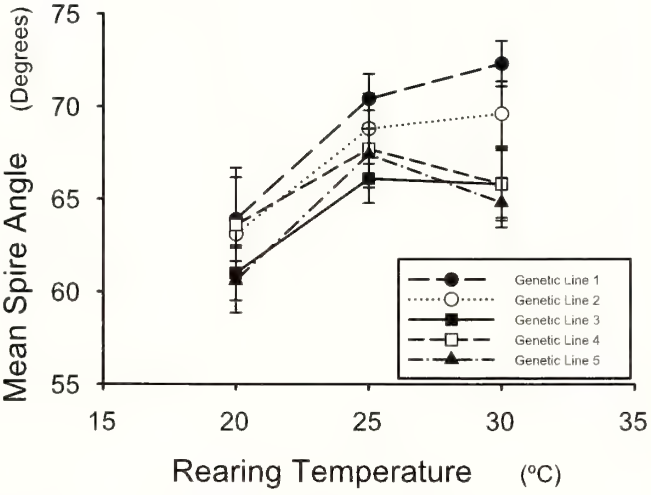

Figure 6. Mean spire angle vs. rearing temperature for specimens of *Physa virgata*. Genetic lines (distinguished by symbols) responded differently to different temperatures. The slopes of lines connecting means represent environmental effects on spire angle while the relative height of the lines represents genetic effects on spire angle. Error bars represent one standard error of the lines represents genetic effects on spire angle. Error bars represent one standard error of the mean.

ACKNOWLEDGEMENTS

We thank Ray Curry, Roberto Gonzales, Valerie Woodard, and Dr. Maj Angarano for assistance in rearing the inbred lines of *Physa virgata*. We also thank Dr. Janice Voltzow, Amy Wethington, and an anonymous reviewer for their critical review and invaluable guidance leading to considerable improvement of this manuscript.

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A 15-year study of interannual shell-shape variation in a population of freshwater limpets (Pulmonata: Basommatophora: Ancylidae)*

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Abstract: A population of an unidentified freshwater basommatophoran, pulmonate, ancylid limpet (subfamily Laevapecinae) inhabiting a natural stream impoundment in southeastern Oklahoma was sampled every fall from 1973 to 1987 and every spring from 1977 to 1988 to assess its shell morphological variation. The population was annual and semelparous, thus, successive fall and spring samples contained individuals from the same generation after 6 and 12 months of growth, respectively. Aperture length (AL), shell height (SH), and aperture width (AW) were measured for all sampled individuals. Analysis of covariance (ANCOVA), with AL as a covariant, indicated significant variation ($P < 0.05$) in AL-adjusted mean AW and SH across fall and spring samples, respectively. ANCOVA tests with AL as a covariant indicated that sample AL-adjusted mean AW and SH varied significantly between spring and fall samples in 4 and 6 of the 11 annual generations sampled, respectively. Shell morphology was not correlated with mean monthly air temperature, total precipitation, or shell growth rate during a six-month period preceding fall or spring samples. Such intrapopulation, interannual, intragenerational variation suggests that shell shape is ecophenotypically labile in freshwater gastropods. Thus, congeneric species designations based on gross shell morphology of type specimens may be problematic, leading to conservation efforts directed at ecophenotypic, shell-shape variants of a common gastropod species rather than truly rare and endangered species.

Key words: Ancylidae, ecophenotypic variation, freshwater pulmonate, intrapopulation variation

The basis of extensive intraspecific, interpopulation variation in molluscan shell morphology has been debated (for reviews see Goodfriend 1986, Trussell and Etter 2001). Diver (1939) ascribed much of intraspecific variation in shell morphology to non-genetic, ecophenotypic plasticity induced by environmental factors such as flow velocity (Lam and Calow 1988, Urabe 1998), substratum type (Eagar *et al.* 1984, Urabe 2000), water borne chemical cues from predators (Appleton and Palmer 1988, Krist 2002), wave exposure (Phillips *et al.* 1973, Crothers 1977, Murty and Rao 1978, O'Loughlin and Aldrich 1987), shore steepness (Yeap *et al.* 2001), shell growth rate (Kemp and Bertness 1984, McMahon and Whitehead 1987, Burnside 1998), food availability (Spight 1973, Burnside 1998), calcium availability (Burnside 1998) or density effects (Kemp and Bertness 1984, Burnside 1998). In contrast, others have suggested that intraspecific variation in shell shape results from genetically-based adaptation of shell form to selective forces such as current velocity (Durrant 1975, Sutcliff and Durrant 1977), wave exposure (Berry and Crothers 1968, Phillips *et al.* 1973, Naylor and Begon 1982, Crothers, 1985), humidity (Emberton 1982, 1995), temperature (Lazaridou-Dimitriadou *et al.* 1994, Parsons 1997), and shell-crushing predators (Johannesson 1986). In other cases, interpopulation variation in shell shape could not be correlated with either non-genetic ecophenotypic influences or selective pressures (Edwards and Humphrey, 1981, McMahon, 1990, 1992, Falniowski *et al.* 1993, Katoh and Foltz, 1994, Lazaridou-Dimitriadou *et al.* 1994, Chapman, 1995, Mukaratirwa *et al.* 1998).

Although the degree of non-genetic ecophenotypic influence on molluscan shell shape continues to be debated, recent research has demonstrated that intraspecific, interpopulation variation in shell morphology cannot be correlated with intraspecific, interpopulation genotypic variation, based on electrophoretic analysis of isozyme polymorphisms. Lack of such correlation is indicative of non-genetic influences on molluscan shell shape (for a review see Trussell and Etter 2001) and has been recorded in almost all such studies of gastropods, including land snails (Falniowski *et al.* 1993, Lazaridou-Dimitriadou *et al.* 1994), marine snails (Edwards and Humphrey 1981, Trussell and Etter 2001) and freshwater snails (Pagulayan and Enriquez 1983, Katoh and

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Foltz 1994, Mukaratirwa *et al.* 1998). Correlation between interpopulation shell morphological and genetic variation has been reported only for the marine intertidal littorine snail *Bembicium vittatum* Philippi, 1846, whose shell morphology was also impacted by non-genetic environmental factors (Parsons 1997).

Further evidence of non-genetic, environmental influence on molluscan shell shape includes loss of the shell morphology unique to isolated populations when individuals are co-reared under constant laboratory conditions (Crothers 1977, 1985, Arthur 1982, Palmer 1985, Lam and Calow 1988, Perry and Arthur 1991, Burnside 1998, Urabe 1998, 2000) or transferred between populations (Nickerson 1972, Hunter 1975, Eagar *et al.* 1984, Etter 1988). Also indicative of ecophenotypic influence is variation in the shell morphology of individuals inhabiting different sites within a contiguous population in both lotic (Durrant 1975, Sutcliff and Durrant 1977, McMahon and Whitehead 1987, McMahon 1990, Katoh and Foltz 1994, Urabe 1998) and lentic habitats (Arthur 1982).

Variation in shell morphology through time within a population has been argued to result from ecophenotypic rather than genetic influences (McMahon and Whitehead 1987, McMahon 1990). Variation in gastropod shell shape occurs over geological time, with morphologies being correlated with environmental conditions (Gould 1970, Williamson 1981, Hayakaze and Chiba 1999). Such temporal variation in shell shape may also occur over much shorter periods. The shell morphology of a population of *Ancylus fluviatilis* (Müller, 1774) changed at the same stream locations over a two-year period (McMahon and Whitehead 1987). Shell morphology varied in three populations of the freshwater pulmonate *Physella virgata* (Gould, 1855) over periods as short as two months (Burnside 1998). Such short-term, temporal, ecophenotypic variation makes identifications of gastropods based solely on gross shell morphological characters such as globosity, aperture shape, and shell ornamentation highly problematic.

To assess better the extent of temporal intrapopulation variation in the shell shape of freshwater gastropods, a population of an unidentified, semelparous, freshwater limpet (Pulmonata: Basommatophora: Ancylidae: Laevapecinae) inhabiting a natural stream impoundment in southeastern Oklahoma was sampled every fall from 1973 to 1987 and every spring from 1977 to 1988. Aperture length, aperture width, and shell height were measured for each sampled individual, permitting analysis of variation in shell morphology among annual generations over a 15-year period and at 2 different periods within the lifetimes of single generations. The impacts of temperature, precipitation, and generation cohort growth rate on temporal morphological variation of shells were also investigated.

METHODS

Specimens were collected from an impounded portion of Bee Branch, a second order tributary of the Mountain Fork River near Beaver's Bend State Resort Park in McCurtain County, southeastern Oklahoma. Bee Branch lies in the southern portion of the Kiamichi Mountains where its drainage area is metamorphic rock in deep ravines. It is highly shaded and relatively oligotrophic with continuous flow. The limpet population was isolated in a small impoundment approximately 20 m in diameter created below a falls (a portable GPS unit determined the impoundment's geographical coordinates to be 34°08.139′N by 94°42.733′W). Limpets were not found in any other stream section despite extensive and repeated searches, suggesting that the impoundment population was geographically isolated. Data recorded at Station 341168/99999 on Broken Bow Lake Dam (National Climatic Data Center, National Oceanic and Atmospheric Administration) approximately 3 km from the collection site revealed that it had a climate typical of the southern-central United States. During the 1973-1989 sampling period mean annual mean air temperature was 16.1°C (s.d. = ±0.59, range = 15.1 to 17.2°C). Mean maximum daily air temperature was 39.5°C (s.d. = ±1.76, range = 36.7 to 42.2°C) and mean minimum daily air temperature was −13.2°C (s.d. = ±3.11, range = −16.7 to −7.8°C). Mean annual total precipitation was 135.4 cm (s.d. = ±23.8, range = 107.8 to 193.6 cm) and mean maximum daily precipitation was 10.6 cm (s.d. = ±1.76, range = 6.4 to 20.7 cm).

The limpet studied could not be identified to species level. It had a fluted, bi-lobed pseudobranch characteristic of the ancylid genera *Laevapex* Walker, 1903 and *Hebetancylus* Pilsbry, 1914 (subfamily Laevapecinae), but did not have the darkly pigmented tentacle core diagnostic for the genus *Laevapex* in North America (Burch 1989). The eccentric apex of the shell pointed to the right of the midline as in *Hebetancylus excentricus* (Morelet, 1851), but the shell was more ovoid and the apex more central than considered diagnostic for that species. In addition, the population fell outside the North American distribution of *H. excentricus*, considered to be southern portions of U.S. states bordering the Gulf of Mexico (Burch 1989). Thus, it could not be identified as one of the described species of North American ancylid limpets at either the genus or species level. Specimens have been sent to the University of Michigan for identification.

Samples (generally $n > 30$) were collected from the sides and undersides of rocks in depths less than 1 m. Specimens were generally restricted to rock surfaces embedded in sand/pebble substrata. To avoid size bias, all specimens on a sampled rock were removed by sliding a scalpel blade gently under the edges of their shells, preventing shell or tissue damage. Rocks were sampled until an appropriate sample size (n > 30) was attained. The population was sampled annually in the fall between September and October from 1973 to 1987 and in the spring between April and June from 1977-1988. All samples were immediately fixed in 10% neutralized formalin and returned to the laboratory for measurement of parameters of shell shape.

The limpet population had an annual semelparous reproductive pattern. Adults oviposited in May/June to produce a cohort that grew through summer and fall to oviposit the following spring. Adults died soon after spring oviposition giving each generation a one-year life span. Individuals collected in the fall had experienced approximately six month's growth from hatching, while those collected the following spring were from the same annual cohort after approximately a year's growth. Spring samples were collected during the oviposition period just prior to hatching of juveniles of the next generation. All annual cohorts described herein are indicated by the year during which they were produced.

The shell aperture length (AL, the greatest anterior-posterior distance across the aperture), aperture width (AW, the greatest distance across the aperture 90° to the anterior-posterior axis), and height (SH, the greatest vertical distance from the shell apex to the plane of the aperture) of each individual were measured to the nearest 0.1 mm with a binocular dissecting microscope at 10X using an ocular micrometer (McMahon and Whitehead 1987) (Fig. 1). AL and AW were measured for specimens submerged in a glass Petri dish. SH was measured using the tip of a fine brush to transfer the specimen from the bottom of the dish onto the side of a vertically mounted glass cover slip to which moistened specimens adhered by water surface tension. In this position the shell was viewed in lateral profile, allowing measurement of SH. Shell morphometric variables were measured for a total of 1,787 specimens.

Data on monthly rainfall and air temperature over the course of the experiment were obtained from annual summaries of climatological data recorded at Station 341168/99999 on Broken Bow Lake Dam (National Climatic Data Center, National Oceanic and Atmospheric Administration), approximately 3 km from the collection site.

ANOVA/ANCOVA and *post hoc* Scheffé's test were utilized to compare differences within spring and fall samples in mean AL, AL-adjusted sample means of AW and SH, AL-adjusted mean AW or SH of spring and fall samples within single generations, and sample shell AL growth versus mean air temperature and total precipitation over the prior six month period. ANCOVA was utilized to generate sample mean SH and AW values adjusted to eliminate the influence of the covariant AL. The relationship between individual AL and AW or SH for all individuals and between shell growth in AL and the AL-adjusted mean AW and SH of spring and fall samples were examined with reduced major axis regression analysis. Least squares linear regression analysis was utilized to examine the correlation between cohort growth in shell AL and mean air temperature or total precipitation over the prior six month period. All statistical analyses were preformed with Statgraphics Plus® version 6.1 (Statistical

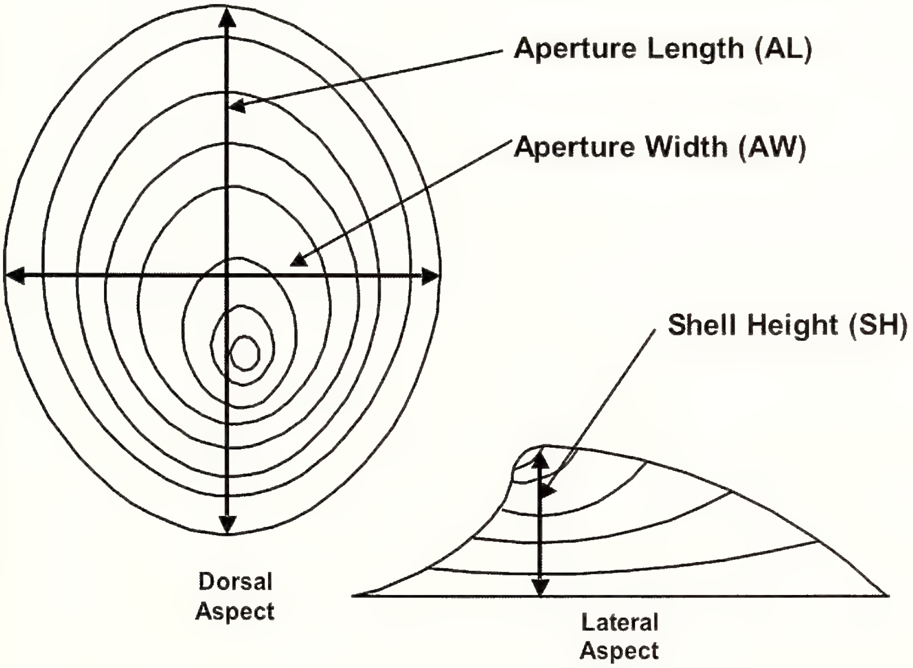

Figure 1. Diagrams indicating shell measurements taken.

Graphics Corporation) with the exception of reduced major axis regression analyses preformed with a program developed by Andrew J. Bohonak of San Diego State University. The α value for all statistical analyses was 0.05.

RESULTS

ANOVA revealed variation in mean aperture length between annual fall ($n = 15$, $F = 41.3$, $P < 0.00001$) and spring samples ($n = 11$, $F = 15.5$, $P < 0.00001$) (Fig. 2). A Scheffé's test allowing multiple pair-wise mean testing indicated that of 105 possible pair-wise comparisons between the sample mean AL values of 15 annual fall collections, 71 (67.6%) were significantly different ($P < 0.05$). Similarly, of 55 possible pair-wise comparisons of mean AL values between the 11 spring samples, 35 (63.6%) were significantly different. Reduced major axis regressions of either AW or SH against AL for all sampled individuals were significant (AW, $a = 0.078$, $b = 0.741$, $n = 1787$, $r = 0.956$, $P < 0.0001$; SH, $a = -0.139$, $b = 0.395$, $n = 1787$, $r = 0.864$, $P < 0.0001$). Regression slope values were significantly less than unity, indicating that relative shell AW and SH decreased slightly with increasing AL (Fig. 3).

When subjected to ANCOVA with individual AL as a covariant to account for size allometry, cohort year had a significant impact on mean cohort SH in both the fall ($n = 15$, $F = 26.29$, $P < 0.00001$) and spring samples ($n = 11$, $F = 35.90$, $P < 0.00001$). A Scheffé's test indicated that 36 (34.3%) of 105 and 28 (50.9%) of 55 possible pair-wise comparisons of AL adjusted mean cohort SH were significantly different in the fall (Fig. 4A) and spring samples (Fig. 4B), respectively. AL-adjusted annual mean SH ranged from 1.07 mm (s.e. = ±0.014, $n = 54$) for the fall 1987 cohort to 1.29 mm (s.e. = ±0.016, $n = 37$) for the fall 1975 cohort (Fig. 4A) and from 1.25 mm (s.e. = ±0.035, $n = 11$) for the spring 1983 cohort to 1.56 mm (s.e. ±.0.015, $n = 62$) for the spring 1979 cohort (Fig. 4B). Similarly, ANCOVA and a Scheffé's test indicated that 49 (46.7%) of 105 and 26 (47.3%) of 55 possible pair-wise comparisons of AL-adjusted mean cohort AW were significantly different in the fall ($n = 15$, $F = 48.22$, $P < 0.00001$) and spring samples ($n = 11$, $F = 17.80$, $P < 0.00001$), respectively. AL-adjusted annual mean AW ranged from 2.48 mm (s.e. = ±0.008, $n = 132$) for the fall 1981 cohort to 2.77 mm (s.e. ±0.015, $n = 54$) for the fall 1987 cohort (Fig. 4C) and from 2.94 mm (s.e. = ±0.031, $n =17$) for the spring 1977 cohort to 3.18 mm (s.e. = ±0.022, $n = 35$) for the spring 1986 cohort (Fig. 4D).

In all cases, mean spring sample AL increased over that of the fall sample AL within any one annual cohort (1977-1987) (Fig. 2), allowing cohort shell growth rate to be estimated as the increase in mean sample AL between the fall and spring cohort samples. Because all individuals in a fall sample of a cohort were produced in May/June, cohort growth after hatching could be estimated as the mean cohort AL of the fall samples. When subjected to reduced major axis regression analysis, increases in mean AL from cohort spring hatching to fall sampling and from fall sampling to subsequent spring sampling versus AL-adjusted mean cohort sample AW or SH as dependent variables proved insignificant (fall AW: $n = 15$, $P = 0.664$; spring AW: $n = 11$, $P = 0.824$, fall SH: $n = 15$, $P = 0.340$; spring SH: $n = 11$, $P = 0.824$).

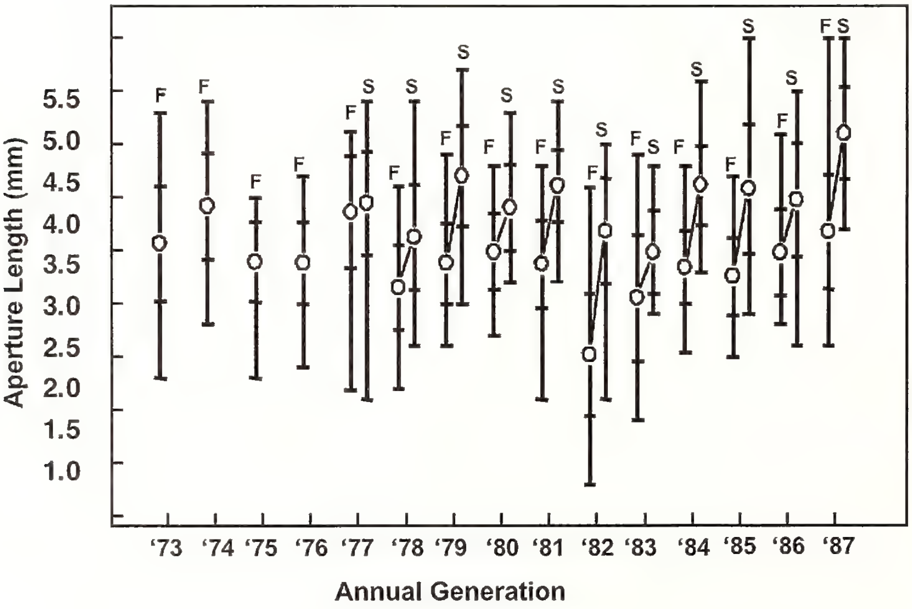

Figure 2. Variation in mean aperture length (AL, vertical axis) of samples of annual generations (horizontal axis) in a population of freshwater limpets. Inner bars about means represent standard deviations of the mean and outer bars the range of AL recorded in the sample. Samples collected during the fall are indicated by an "F" at the upper end of the error bar and those collected in the spring by an "S." Paired fall and spring samples of the same annual cohort are connected by solid lines (cohorts '78 through '87). Mean fall cohort values reflect the increase in AL in the first six months of life, while the difference in mean AL between fall and subsequent spring samples of cohorts represents the mean increase in cohort AL in the subsequent six months of life in this semelparous, spring-ovipositing species.

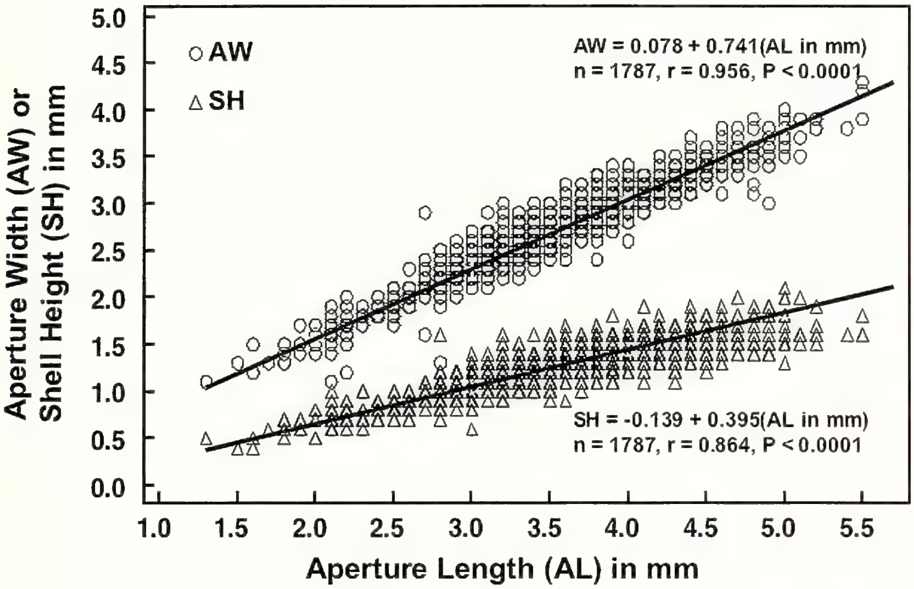

Figure 3. Relations of shell aperture width (AW, circles) and shell height (SH, triangles) (vertical axis) to aperture length (AL, horizontal axis) in all sampled individuals (n = 1787) of a population of freshwater limpets collected from 1973-1988. Data points for all 1787 measured individuals do not appear in this figure due to extensive point overlap. Solid lines are best fits of significant ($P < 0.0001$) reduced major axis regressions. Regression equations relating AW and SH to AL and associated statistical parameters are indicated above the AW data and below the SH data, respectively.

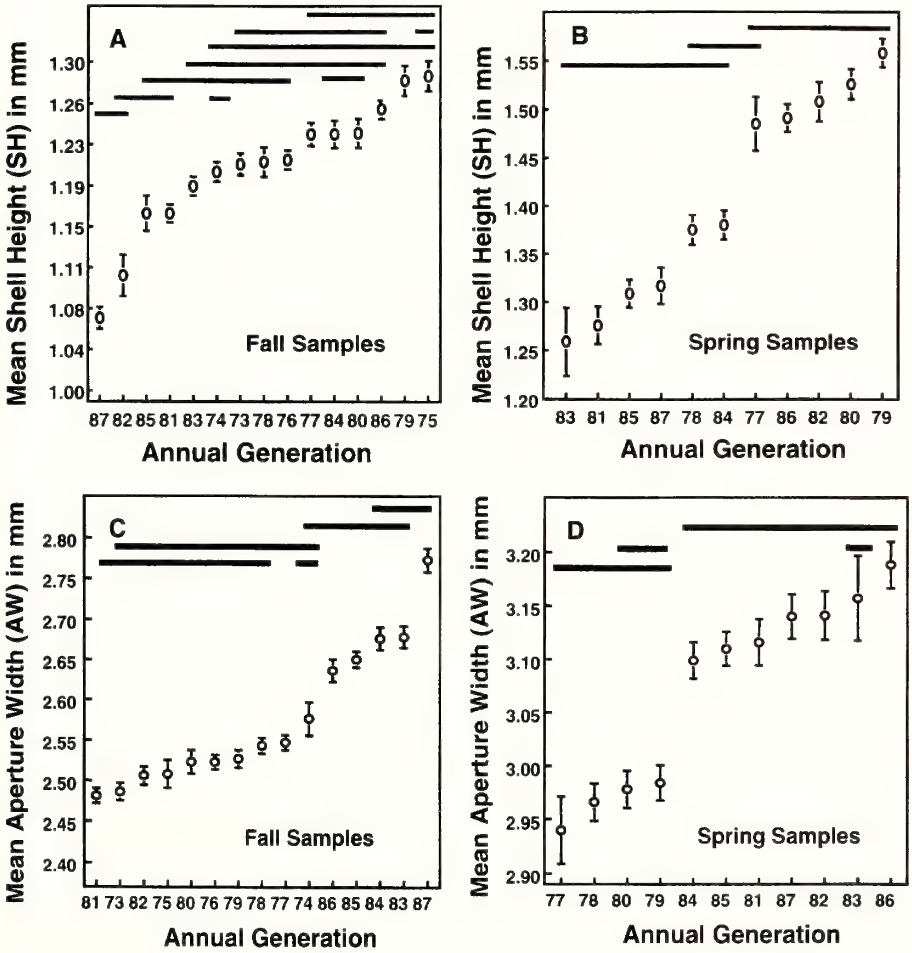

Figure 4. Aperture length-adjusted mean shell height (SH) and aperture width (AW) (vertical axis) of samples of annual cohorts (year of cohort formation indicated on the horizontal axis) of a population of freshwater limpets. A. Adjusted mean SH of cohort samples collected during the fall. B. Adjusted mean SH of cohort samples collected during the spring. C. Adjusted mean AW of cohort samples collected during the fall. D. Adjusted mean AW of cohort samples collected during the spring. Vertical bars about the means represent standard errors of the means. Horizontal bars in the upper portion of the figures encompass means that were not significantly different.

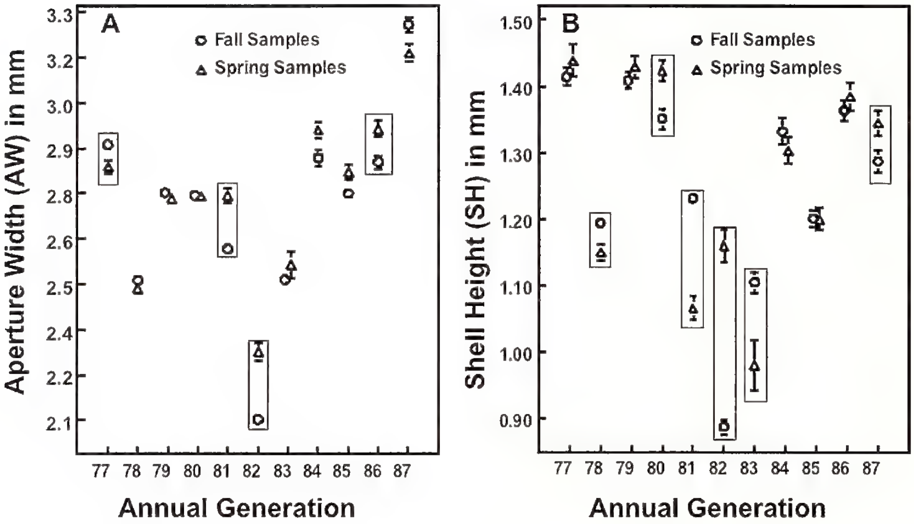

Figure 5. Aperture-length adjusted mean aperture width (AW) and mean shell height (SH) of paired spring and fall samples of the same annual cohort (indicated by date of cohort formation on the horizontal axes) of a population of freshwater limpets. Open circles and triangles represent the adjusted mean AW (A) or SH (B) of fall and spring annual cohort samples, respectively. Error bars represent standard errors of the mean. Standard error of the mean was less than symbol width for means without error bars. Paired annual cohort values of mean AW (A) or SH (B) enclosed within boxes were significantly different from each other based on a Bonferroni correction of α = 0.05 for sequential tests.

The difference between AL-adjusted mean AW and SH of fall and spring samples within a specific annual cohort was tested by ANCOVA with fall and spring sample periods as the treatment and AL as the covariant. Application of a Bonferroni correction for sequential tests to α = 0.05 indicated that the mean AW of the fall sample was significantly different from that of the spring sample in 4 (36.4%) of 11 paired annual cohort fall and spring samples (i.e., the 1977, 1981, 1982, and 1986 cohorts, Fig. 5A). Similarly, the mean SH of the fall sample was significantly different from that of the spring sample in 6 (54.5%) of 11 paired annual cohort fall and spring samples (i.e., the 1978, 1980, 1981, 1982, 1983, and 1987 cohorts, Fig. 5B). Variation in mean AW and SH between fall and spring cohort samples was nondirectional. Mean AW was significantly lower in spring compared to fall samples in the 1977 cohort and significantly higher in the 1981, 1982, and 1986 cohorts (Fig. 5A). The mean SH of the spring sample was significantly lower in the 1978, 1981, and 1983 cohorts and significantly higher in the 1980, 1982, and 1987 cohorts (Fig. 5B)

ANOVA of cohort increase in mean AL between fall and spring samples (i.e., overwinter) indicated that neither average air temperature (n = 11, P = 0.081) or total precipitation (n = 11, P = 0.459) significantly impacted shell growth rate measured as increase in AL. In contrast, ANOVA indicated that growth of annual cohorts between time of hatching and fall sampling (i.e., over summer) was positively correlated with both average air temperature (n = 15, P = 0.035) and total precipitation (n = 15, P = 0.05). Because air temperature and precipitation levels potentially affected shell size, the relationship between either mean air temperature or total precipitation during the preceding six month growth period and AL-adjusted mean cohort sample AW or SH was investigated with least squares linear regression analysis. Application of a Bonferroni correction to α = 0.05 for sequential tests to these regression analyses indicated that neither sample mean SH or AW was significantly correlated with mean air temperature or total precipitation over the six month period prior to collection in either fall-collected or spring-collected samples.

DISCUSSION

Malacologists have been concerned about the impact of nongenetic, ecophenotypic variation in shell morphology on molluscan taxonomy and systematics, where species designations can depend entirely on subtle differences in shell shape or ornamentation (Diver 1939). If shell morphology is ecophenotypically plastic, species identifications based solely on the shell morphology of type specimens may lead to the naming of multiple species in geographically separate populations that are environmentally-induced variants of a single, geographically widespread, ecophenotypically variable species. Sixty five populations of the freshwater pulmonate genus *Physella* Haldeman, 1842 from Texas and adjacent states displayed extensive interpopulation shell-shape variation which did not cluster around any of the 60 species and subspecies of the genus described as occurring in North America (Burch 1989), suggesting that *Physella* potentially represents a single species with a highly ecophenotypically plastic shell morphology (Burnside 1998). This result was supported by the observation that North American *Physella integra* (Haldeman, 1841) and *Physella heterostropha* (Say, 1817) were capable of interbreeding with each other and with European *Physa acuta* (Draparnaud, 1805) without reduction in hybrid fecundity, suggesting that they were ecophenotypic variants of a single species (Dillon *et al.* 2002).

Variation in shell shape that occurs among individuals sampled from different localities within single interbreeding gastropod populations or is lost when individuals are co-reared under constant laboratory conditions or transferred into other habitats (see Introduction) is almost certainly the result of non-genetic, ecophenotypic influences. Urabe (1998) found 0% heritability of basic shell-shape differences in the freshwater "prosobranch" *Semisulcospira reiniana* Brot, 1877. Lack of heritability of shell morphology also has been reported in *Physella virgata* (Burnside 1998).

While the ecophenotypic influence on interpopulation variation in the shell morphology of gastropods has been well documented, there have been relatively few studies of temporal variation. Three of six European populations of the stream limpet, *Ancylus fluviatilis*, exhibited changes in shell morphology over a period of two years (McMahon and Whitehead 1987) and shell globosity and relative aperture roundness varied significantly in populations of *Physella virgata* within periods as short as two months (Burnside 1998). My study confirmed the potential for extensive intrapopulation, short-term temporal variation in shell shape in freshwater gastropods and, presumably, gastropods in general. Shell size measured as AL varied among fall and spring samples of annual cohorts with 67.6% and 63.6% of possible pair-wise comparisons of fall and spring samples being significantly different, respectively. AL-adjusted mean SH significantly differed between 46.7% and 47.3% and mean AW varied between 34.3% and 50.9% of possible pair-wise comparisons among annual fall and spring cohorts, respectively. AL-adjusted mean SH of fall cohort samples ranged from 1.07 mm (1987) to 1.29 mm (1975), equivalent to 10.8% lower and 7.0 % greater than the overall 1.20 mm mean value for all annual samples, while, spring cohort sample mean SH ranged from 1.32 mm (1987) to 1.49 mm (1977), equivalent to 6.4% lower and 5.7% higher than the 1.41 mm overall mean for all samples. Similarly, mean fall cohort sample AW ranged from 2.49 mm (1973) to 2.77 mm (1987), equivalent to 3.4% lower and 7.4% greater than the 2.58 mm overall mean for all samples, while mean spring cohort sample AW values ranged from 2.94 mm (1977) to 3.14 mm (1987), equivalent to 4.5% lower and 1.9% higher than the 3.08 mm overall mean for all samples.

Somewhat surprisingly, the extent of interannual variation in shell shape in this study approached that occurring among geographically separated populations of other ancylid species. Thus, mean SH adjusted for a 4.5 mm AL individual among 31 populations of the freshwater limpet *Ancylus fluviatilis* was 2.05 mm with a range of 1.89 to 2.22 mm, equivalent to 7.8% below and 8.3% above the overall mean for all samples (McMahon and Whitehead 1987). Similarly adjusted mean AW in these populations was 3.45 mm with a range of 3.26-3.57 mm equivalent to 5.5% below and 3.5% above the overall mean for all samples (McMahon and Whitehead 1987). Based on shell morphometric data for 16 populations of the North American stream limpet, *Ferrissia rivularis* (Say, 1817) (Nickerson 1972), mean SH adjusted to an individual with an overall mean AL of 3.5 mm was 1.16 mm with a range of 1.01 to 1.27 mm equivalent to 12.9% below and 9.5% above the overall mean SH for all samples. Adjusted mean AW among these populations was 2.36 mm with a range of 2.13-2.73 mm or 9.7% below and 11.6% above overall mean AW (Nickerson 1972).

Thus, the data presented indicate that the implicit assumption that shell shape is relatively stable within populations may be spurious and requires further investigation. My data indicate that temporal variation in shell morphology within a population of gastropods can be extensive and approaches that recorded among geographically separated populations. This result suggests that species designations of freshwater pulmonates (and possibly any gastropod species) should not be based solely on the shell morphology of the type specimen, as shell parameters can be ecophenotypically variable rather than genetically fixed diagnostic characteristics. Thus, within-genus species designations based on minor differences in gross shell morphology could lead to a shell-shape ecophenotype of a wide-spread, common species being misidentified as a rare, geographically isolated species. Such misidentifications could lead to conservation efforts and funds being directed at environmentally-induced ecophenotypes of common species rather than truly rare and endangered species.

In my study, the potential for extensive temporal variation in the shell morphology of freshwater gastropods was exemplified by the significant and non-directional change in relative AW and SH recorded through the life span, respectively, in 4 and 6 of the 11 annual generational cohorts for which paired fall and spring samples where taken. Such short-term, random variation in shell shape through time within a single generation may account for observations of intrapopulation variation in the shell morphology of samples of three populations of *Physella virgata* within a period of just two months (Burnside 1998).

Shell growth rate can influence shell shape in gastropods. The shell shape of *Ancylus fluviatilis* was related to shell growth rate with snails from populations with faster growing shells having relatively greater AL and reduced SH, leading to a flattened, more rounded patelliform shell. Similarly, faster growing individuals of the marine intertidal littorine *Littorina littorea* (Linnaeus, 1758) developed more globose shells (Kemp and Bertness 1984). In contrast to these results, shell growth rate in the studied limpet population was not correlated with relative AW or SH. Lack of correlation between shell growth rate and shell shape also occurred in the marine littorinid, *Bembicium vittatum* (Parsons 1997) and the freshwater pulmonate, *Physella virgata* (Burnside 1998).

The relative AL and SH of both fall and spring samples of the studied limpet population were not correlated with average air temperature or total precipitation over the six month period preceding collection. Lack of correlation with either parameter may have been due to a multiplicity of unidentified environmental factors interacting synergistically or antagonistically to generate the apparent random intra-annual and inter-annual shell morphological variation reported in this study. The interactive impacts of environmental factors on gastropod shell morphometry require further field and laboratory investigation before the bases of intra-population and inter-population variation in gastropod shell shape can be understood.

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Leopold von Buch's legacy: Treating species as dynamic natural entities, or why geography matters

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Abstract: Although not unknown in the 19th century, but underutilized in its modern sense, the idea of using reproductive isolation (or its converse, the potential to interbreed) as a criterion to define species and to distinguish species taxa from one another goes back to the German naturalist, geologist and palaeontologist Christian Leopold Freiherr von Buch [1774-1853]. As a "Darwinian before Darwin" he perceived reproductive isolation as the defining property of species and adopted for the first time (1) the non-essentialistic concept that species are not types but populations consisting of individuals or groups of such populations and (2) a model of geographic speciation. During the 19th century, which was dominated by essentialism, creationism, and a typological species concept, few naturalists grasped the importance of this concept, among them the malacologist Albert Mousson [1805-1890]. Primarily as a consequence of Ernst Mayr's synthetic works of 1942 and 1963 reproductive isolation became widely accepted as being the most valuable concept for defining biological species and the notions of geographical variation, separation, and the non-applicability of the degree of (phenotypic) distinctness became part of the biological species concept. The last two decades have seen an active phase of debate about how to define and delineate species. Emphasizing the often neglected but instructive historical perspective, the present paper briefly reviews developments leading to the wide application of the Biological Species Concept (BSC) during the mid 20th century, and contrasts and discusses this concept within the context of malacology. Although the BSC has been challenged conceptually and operationally on the basis of being non-universal, non-dimensional (i.e. horizontal only), and operationally impractical, it is argued here that alternative suggestions—such as the Evolutionary Species Concept (ESC) and especially the recently much-favored Phylogenetic Species Concept(s) (PSC)—are arbitrary (with nebulous and vague definitions), artificial and reductionistic (non-biological) and operation-oriented (serving only diagnosability), as was the 19th century typological approach, which resulted in a ballooning of species numbers. It is discussed why diagnosability is not a sufficient criterion for a species definition and argued that the PSC describes species taxa rather than defining a species concept. Outlining the species concept debate and based on this important distinction of species concepts (defining species) versus species taxa (describing species), it is concluded that the difficulties of applying the BSC are not sufficient to justify its rejection in favor of other, logically and biologically inferior concepts. Accordingly, the BSC should be favored over the PSC(s) because it is the only definition that provides an objective criterion, reproductive isolation, and is primarily based on the biological significance of species. In addition, for the demarcation of species taxa, morphological, molecular, geographic, ecological, and behavioral information should be inferred in order to rank geographically isolated populations as species or subspecies. In this context, two case studies from malacology, that utilize freshwater gastropods of the former "melaniid species basket" (Paludomidae and Pleuroceridae) are used to advocate the conception of species as representing dynamic entities in a historical and geographic context.

Key words: Species concepts, species taxon, biological species, phylogenetic species, typology, freshwater gastropods, Paludomidae, Pleuroceridae

> "When I use a word," Humpty replied, in a scornful tone, "it means just what I choose it to mean—nothing more nor less." "The question is," said Alice, "whether you can make a word mean so many different things." "The question is," said Humpty, "who is to be master, that's all." (Carroll 1865).

Species are of paramount importance for biology. The species is not only the fundamental concept for systematists and the basic unit for ecology, species is also a basic unit of the real world, and thus an evolutionary and dynamic entity, providing the units of diversity and "the coin of evolutionary change" (Mayr 1988a, 1991, 1997: 134), even for those authors criticizing various aspects of the modern synthetic theory of evolution (*e.g.* Gould 2002). The frequent observation that the fauna of a given region is not a chaotic assemblage of intergrading individuals of all kinds lends support to the conviction of the existence of non-arbitrary discontinuities in nature to be called species. Only with this assumption taken as a fact do the many researches into the details and mechanisms of the speciation process become meaningful. In addition, for any assessment of the world's biological diversity (for a recent malacological example see Bouchet *et al.* 2002), a sound judgment on what to consider a species is a *conditio sine qua non.*

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It has been ignored for centuries that in contrast to other natural sciences such as chemistry and physics, biology is not striving in the same way to detect natural laws. While theories in the physical sciences are usually based on laws, those in biology are based on concepts. Biology, as all life sciences, attempts to find concepts that explain the natural world. Arguing for biology as an autonomous science and against the common attitude which favors discoveries and experiments over concepts, it is Mayr (1997: 26) who pointed out that today concepts such as evolution, natural selection, sexual selection, competition, altruism, or the gene "are as significant in biology as laws and discoveries are in the physical sciences and yet their importance was strangely ignored until quite recently." This is not saying, of course, that the discovery of new facts and observations are irrelevant, but rather that the major contributor to a new theory in life sciences is the development of new concepts. Changes in concepts (e.g. natural selection as an agent of evolution) had and still have much more impact in biology than new discoveries, as is best illustrated by the two so-called "Darwinian revolutions" (see Mayr 1991). As much as a scientific theory is not to pronounce a truth but to generate hypotheses to be tested, a concept tries to synthesize all the facts and evidence available at a given time (see also Murphy 2002).

Species as a concept is one of the most debated issues of evolutionary biology and phylogenetic systematics. With a plethora of species concepts and definitions proposed and disputes evoked, few questions in science have elicited such a long-standing and heated debate as the "species problem" (for example Dobzhansky 1935, 1937, Mayr 1942, 1957, 1963, 1988a, 1988b, 1991, 1996, 1997, 2001, Simpson 1961, Hull 1965, Peters 1970, Gittenberger 1972, Griffiths 1974, Mishler and Donoghue 1982, Cracraft 1983, 1989, 2000, Sudhaus 1984, Reif 1984, Vrba 1985, Willmann 1985, 1991, Haffer 1986, 1991, 1992, 1995b, 1998, Iwatsuki *et al.* 1986, Häuser 1987, Mishler and Brandon 1987, De Queiroz and Donoghue 1988, McKitrick and Zink 1988, Otte and Endler 1989, Frost and Hillis 1990, Nixon and Wheeler 1990, Sluys 1991, Haffer 1992, O'Hara 1993, 1994, De Queiroz 1995, Claridge *et al.* 1997, Ghiselin 1997, Eck 1998, Howard and Berlocher 1998, Wilson 1999, Wheeler and Meier 2000, Hey 2001a, 2001b, Schilthuizen 2001, see also book reviews by Avise 2000b, Hull 2002, Shaw 2002, Wake 2002). What seems to be just a semantic battle for some is the central ontological problem of systematic biology for others. In the views of some taxonomists at least, there is "already too much literature" on this problem and " it has been discussed, argued over, and symposiumed to death for years" (Winston 1999: 43). In addition, it has been claimed several times by various authors, either in arrogance or ignorance that they have found the final solution to the species problem (e.g. Ghiselin 1975, Vrana and Wheeler 1992). However, because species concepts are mostly explicitly linked to mechanisms of speciation (e.g. Chandler and Gromko 1989), discussing these concepts has profound implications for evolutionary theory and, thus, are more than just an off-side battle over words.

Since the early 19th century, at hardly any period has there been agreement over the species question or on a single accepted definition of what constitutes a species. Although for about half a century the Biological Species Concept (BSC) was widely accepted in theory and practice, with the cladistic view increasingly dominating systematic biology during the last two decades, many realized an urgent need to re-evaluate relevant and related concepts in this context. It is neither possible nor intended in the present paper to cover this ongoing debate comprehensively. Given that species are so important for evolutionary biology, systematic phylogeny, classification, and taxonomy, and that the pros and cons of the debate have not always found adequate consideration in malacological literature, though, a very brief *tour d'horizont* is attempted here with some examples from freshwater gastropods added as illustrations.

Although suggested by the title of his epic work "On the Origin of Species," it was not Charles Darwin (1859) who taught us first about the nature of species. Naturalists such as George-Louis Lecerc, Comte de Buffon [1707-1788], and Johann Friedrich Blumenbach [1752-1840], during the century before Darwin had already a fairly good perception of species based on isolation. However, their concept was based on typology, assuming the infinite existence of fixed species boundaries and a constant type (see Lenoir 1980, 1981, Ghiselin 1969, Mayr 1982, Rheinberger 1990, J. Haffer pers. comm.). Thus, the idea of reproductive isolation, or its converse, the potential to interbreed, as criterion for species discrimination was not unknown, but underutilized in our modern sense during the 19th century and the first half of the 20th century. Its modern perception in the context elaborated below goes back to the German naturalist, geologist, and palaeontologist Christian Leopold Freiherr von Buch [1774-1853]. In his accounts on the natural history of the Canary Islands, Buch (1819, 1825) perceived the criterion of reproductive isolation as the defining property of species. If one follows the century-long debate of the species problem, one gets the impression that it has been little appreciated how strongly natural processes such as the formation of species are constrained by the necessity of having to take place in a three-dimensional space. Therefore, following

Buch's original insight, I will emphasize here why geography matters as a key factor in the context of the species debate.

TYPOLOGY IN MALACOLOGY

Typological thinking, or essentialism, has long dominated systematic biology and particularly taxonomy, including paleontology (Mayr 1957, 1963, 1982, 1988a, 1997, Hull 1965, Griffiths 1974, Willmann 1985, McOuat 2001, Haffer 1997a, 1997c). For the typologist, the type (the unchanging *eidos*, here not to be confused with the type as instrument of taxonomic procedure regulated by nomenclature) is real and the variation an illusion. In contrast, for modern evolutionary biologists used to thinking about populations, the type is an abstraction (a mere statistical average or arbitrarily chosen specimen for taxonomic purpose) and only the variation is real. The typological species concept forced naturalists to consider as species different variants within a population and to recognize as full species those local populations that differed by one diagnostic character from other populations. Consequently, because this concept was entirely consistent with the belief in creationism, typology precluded any belief in descent with modification of the Darwin-Wallace theory of evolution, which replaced the idea of *eidos* from Plato's philosophy with variable populations in nature. In contrast, the typological approach to nature corresponded to morphologically delineated species in quite the same way as the recent revived focus on diagnosability does within the framework of the phylogenetic species concept (see below). For a detailed introduction on the history of typology see Mayr (1982, 1988a, 1991, 1997), and Haffer (1997c). For its role in palaeontology see Willmann (1985) and Reif (1997).

In malacology, a typological tendency prevailed for centuries. For a long period malacology was essentially not much more than "conchology," the description and naming of shells, instead of a biological discipline. This is not to deny the valuable contributions in describing nature's diversity by distinguished practitioners during what has been called the "Golden Age" of malacology (see Lindberg 2002). However, typological thinking led to focussing on the often wordy but hardly practicable descriptions of minor conchological differences of single or few individual shells instead of the characteristic features and biological properties of larger series from populations in their geographical and/or ecological context. Such thinking dominated the malacological literature not only for most of the 19th century, but continued into more recent times.

As a consequence, this restriction to conventional typology long hampered modern approaches in malacology. This stands in contrast to ornithology, for example, where proponents of the "New Systematics" transformed the discipline into a fully biological science that was then instrumental in the development of a synthesis in evolutionary theory developed during the first half of the 20th century (reviewed in Mayr 1982, Reif *et al.* 2000). With the full employment of the Darwin-Wallace theory of evolution and the study of geographical variation (the "geographical principle," as exemplified in Glaubrecht 2002), naturalists realised that (1) types (or essences) do not exist in the living world, and that (2) species are composed of populations that vary from location to location and whose individuals vary within a given population. Prior to this realization, the typological method applied in malacology led to the descriptions of a plethora of new taxa, especially species and genera, resulting in today's unmanageable taxonomic-nomenclatorial cornucopia that is illustrated in the extensive synonymies. Among the many examples, see the synonymy and taxonomy of *Littorina* Férussac, 1822 in Reid (1996) or those of some of the diverse taxa within the so-called freshwater "melaniids" (Brot 1874, Starmühlner 1976, Köhler and Glaubrecht 2001, 2002) that long prevented any meaningful phylogenetic and/or biogeographic analysis.

For this reason it would be good practice for any modern taxonomic and systematic account on molluscs to start with an evaluation of whether the specific taxa dealt with actually represent biological entities independent of human perception or instead are mere phantoms of this perception. However, most authors have for a long time worked without paying much attention to the theory of species concepts and to practical implications, more so in malacology than in other, theoretically more advanced disciplines, such as ornithology. This led to the two general trends still pertinent to systematics not only of molluscs: Either (1) assigning species names and status even to populations characterized by trivial conchological characters, insignificant morphological features, and/or low genetic differentiation or (2) merging taxa into a single species or, above the species level, into species complexes and rarely superspecies (as advocated here and explained below). These trends are exemplified in studies on limnic gastropods, for example by Hubendick (1951), Davis (1981), Ponder *et al.* (1994), and Wilke *et al.* (2000) for hydrobiid snails, by Glaubrecht (1993, 1996) for the Mediterranean cerithioidean genus *Melanopsis*, and for SE Asian taxa of the pachychilid genus *Brotia* H. Adams, 1866 *sensu lato* by Köhler and Glaubrecht (2001, 2002, 2003). This unresolved situation is certainly not rendered less complicated by the application of various molecular genetic techniques, as is revealed in studies of different evolutionary lineages, for example, among Hawaiian tree snails of the Achatinellinae (Thacker and Hadfield 2000), North American freshwater Pleuroceridae (Holznagel and Lydeard 2000), or limnic bivalves such as *Potamilus* Rafinesque, 1818 (Roe and Lydeard 1998), *Fusconaia* Simpson, 1900, *Obovaria* Rafinesque, 1819, and *Quincuncina* Ortmann, 1922 (Lydeard *et al.* 2000), and *Corbicula* Megerle von Mühlfeld, 1811 (Pfenninger *et al.* 2002).

The arbitrariness of the widely used approach of trying to keep distinguishable populations taxonomically apart even when based on insufficient morphological characters is illustrated by the case of the freshwater pulmonate genus *Physa.* Recent findings of Dillon *et al.* (2002) considerably helped to free the confused systematics and taxonomy of American Physidae with their currently recognized c. 40 "species," many of which reveal variable and overlapping morphologies, from taxonomical (i.e. nomenclatorial) baggage. The authors were unable to detect evidence of reproductive isolation among six populations from North America and Europe of *Physa heterostropha* (Say, 1817), *Physa integra* (Haldemann, 1841), and *Physa acuta* Draparnaud, 1805, which, therefore, should be combined under the latter species name. In another example, Falniowski and Wilke (2001) reported for (partly allopatric) populations of the two named European species *Marstoniopsis scholtzi* (A. Schmidt, 1856) and *Marstoniopsis insubrica* (Küster, 1853) extremely low genetic divergence and a lack of morphological differentiation, suggesting that all populations belong to only one species, *M. insubrica.*

On the other hand, several endemic radiations of hydrobiid snails (where the problem of species discrimination are compounded because of the scarcity of complex or quantitative characters), have been studied in the last decade for Australian taxa from artesian springs in western Queensland (Ponder and Clark 1990), from artesian springs in northern South Australia (Ponder *et al.* 1989), from Tasmania and eastern Victoria (Ponder *et al.* 1993, Ponder *et al.* 1994), as well as from springsnails in arid regions in south-western North America (Hershler and Sada 1987, Hershler and Landye 1988, Hershler 1989). In all of these cases, the authors named many previously undescribed taxa as distinct species.

Because one of the problems with the species question, in my opinion, is that we constantly lose the ground that was already conquered by others, the present paper will approach this debate from a historical perspective. We forget about the achievements of earlier authors, their ideas, approaches, and concepts that are manifest in part of the literature often erroneously regarded anachronistic, leading to the impression that each generation of biologists "re-invents the wheel" instead of modifying earlier usage. Therefore, the following account gives an overview of some of the more important disparate concepts and definitions of what species are and discusses criteria for choosing a particular species concept. Certainly by not more than a curious coincidence, my own research on limnic gastropods focuses on the same questions and phenomena Leopold von Buch pointed out so early on. Thus, adding to the historical and theoretical aspects, I will present some of the findings on freshwater Cerithioidea and expand on the debate by introducing additional aspects to illustrate how to deal with multidimensional species from an evolutionary biologist's perspective.

LEOPOLD VON BUCH'S SPECIES CONCEPT

In early April 1815 the Prussian geologist and naturalist Leopold Freiherr von Buch started an eight-months' journey to the Canary Islands, located off the coast of northern Africa (for a biography see Günther 1900). It was his plan to study the natural history of this archipelago. Although a geologist by profession and, thus, interested in the vulcanism of the islands, Buch's other special interest was botany. During 1815 he studied the endemic and the non-endemic plants and the vegetational differences on the various islands in the Canaries. Two years after this journey, in November 1817, Leopold von Buch gave a lecture at the Prussian Academy of Sciences in Berlin, reporting on the flora of the Canaries and discussing the relations of the vegetation of this archipelago with that of the African mainland and Europe (Buch 1819). In this context, and in his later book-length account (Buch 1825), he also reflected on a central biological subject, the manner in which the stem species of a genus becomes divided into separate species. It is this discussion that makes him a true "Darwinian before Darwin," as he was called by Günther (1900). Jürgen Haffer (pers. comm.) reviews the history of the biological species concept and discusses in some detail Buch's research and his significance as the founder of the biospecies concept and as the first exponent of the theory of geographic (allopatric) speciation.

Buch (1825: 132-133) wrote: "The individuals of a genus spread out over the continents, move to far-distant places, form varieties (on account of differences of the localities, of the food, and the soil), which owing to their segregation cannot interbreed with other varieties and thus be returned to the original main type. Finally these varieties become constant and turn into separate species. Later they may reach again the range of other varieties which have changed in a like manner, and the two will now no longer cross and thus they behave as two very different species" (cited from the translation in Mayr 1942: 156; see also Mayr 1963: 483 and Kottler 1978: 285). This is the earliest brief yet clear discussion of what was later known as the biological species concept of the modern synthesis of evolutionary biology.

Buch's definition is the most original theory of species and speciation of the early 19th century, even by choosing a few different terms we can hardly improve on his central tenet and have the BSC as it was defined more than a century later. In analysing the essential features of Buch's definition we find that, first, it is a non-essentialistic concept. Buch considered species not to be types but populations or groups of populations consisting of biologically unique individuals. Thus, unusual for his time, which was dominated by typological thinking, he shifted to population thinking long before Darwin laid the foundation with his new concept of natural selection. Buch defined species not in terms of degree of morphological differences but rather stressed that they are separated by a (bridgeless) reproductive gap. And, second, he suggested a model of geographical speciation, starting from the notion of geographical variation without fixed species limits and the idea that geographical isolation is needed to permit species differences to "become constant."

LEOPOLD VON BUCH'S LEGACY

Leopold von Buch's (1819) first publication of his critical definition of species and his theory of speciation later fell into oblivion. However, in 1825 he published a large book on the natural history of the Canary Islands (Buch 1825) which, in a French translation (1836), was read, for example, by Charles Darwin [1809-1882] and Alfred Russel Wallace [1823-1913]. Darwin mentioned the "admirable discussion" of Buch in his Notebook B of 1838 (Kottler 1978), while Wallace, having read and appreciated Buch's book, translated the same critical paragraph into his "Species Notebook" in 1857 (Beddall 1968, Kottler 1978). Without a doubt, he fully appreciated the observation on geographical isolation given his own experiences in the Indo-Malayan Archipelago. However, like Darwin, Wallace focused mainly on how selection drives evolution over time, and not so much on the mechanism of (geographical) speciation.

Albert Mousson [1805-1890] of Zurich, Switzerland, a well-known malacologist who had a personal connection to Leopold von Buch as a student (for a brief biography see Meier 1993), devoted an entire introductory chapter in his book on the "Land and Freshwater snails of Java" to the concept of species in conchology. There he defined species as "all normally formed individuals which interbreed fully fertile with one another" and as "the total of individuals, interconnected by descent and reproduction, maintaining unlimited reproductive capabilities" (Mousson 1849: 3). Way ahead of his time, he also stated that "nature provides us as independent organisms only with individuals, which are comprised by the collective name of the species. Nevertheless, this concept is not a mere abstraction, but has a certain reality in nature" (1849: 2, my translation). Mousson emphasized species as objective entities, not just as artifical units. He distinguished species clearly from subjective categories such as genera, family, and order, and he used, following Buch, reproductive isolation as the decisive criterion for the assignment of species status. In his opinion, naturalists should not create species, but detect them. By recognizing the geographical variation potentially leading to speciation, Mousson also gave up the typological view of fixed species limits. Interestingly, although Mousson's collection comprised about 200,000 shells of about 8500 species and subspecies, of which he described 450 as new, he never designated a single specimen as "type" and, therefore, his collection only consists of type series (Meier 1994). Thus, although a malacologist was among the first to suggest the application of reproductive isolation for a species definition, Mousson's insight was lost for over a century in malacology. As pointed out by Giusti and Manganelli (1992), who cited some examples from those who work with terrestrial and limnic molluscs, in everyday practice the typological approach (i.e. regarding as species only what can be morphologically defined) is still widely used as if Mousson was never read or understood.

A couple of 19th century naturalists, among them Henry Walter Bates in England, Moritz Wagner and Ernst Haeckel in Germany, and Benjamin Walsh and John Thomas Gulick in America, also viewed species as interbreeding populations, but they never developed this theme in further detail (Haffer 1992). For example, the naturalist-missionary Gulick [1832-1923], who studied the Hawaiian tree snails of the family Achatinellidae, became not only a critic of deterministic adapationism, but also an early advocate of the importance of isolation in the generation of new species (Reif 1985). He later stated that "the soundness of the claim that the prevention of free crossing is a necessary principle in the divergent evolution of races and species" gained wide recognition among biologists of the early 20th century (Gulick 1905: 51). However, it was the publications of Rensch (1929, 1934) and Mayr (1940, 1942) that established speciation and the criteria involved in a biological species concept in the most comprehensive way (see Mayr 1963, 1982, Haffer 1992).

WHY DO SPECIES MATTER?

In the context of the question of what species are, it is certainly not helpful that the word "species," deriving from Latin "specere," means "to see" or "to recognize." However, species are more than what a biologist recognizes. Because the discussion of no other concept in biology is hampered more by misunderstanding and confusion of terms, a careful application of terminology (i.e. use of language) is a prerequisite in this debate, as was pointed out explicitly by Ax (1984), Ghiselin (1984), Mayr (1988a, 1997) and Wägele (2000). Constructive discussions can only be carried out based on clearly defined concepts. The debate about species, in my opinion, has not seldom been approached with the dangerous attitude of at best neglecting, at worst ignoring, the admittedly vast amount of literature and its theoretical, biological, and philosophical implications.

Unfortunately, it is impossible to review here some of the indispensable theoretical aspects of the species question, for example whether species are artificial and arbitrary constructions or have an actual existence in nature (see Burma 1949, Ghiselin 1966, 1975, 1997, Löther 1972, Griffiths 1974, Hull 1976, Willmann 1985, 1991, Mayr 1988a: 335-358, Nelson 1989, Mallet 1995, Bock 2000). The present paper assumes *a priori* that species are real, that is, natural and evolutionary entities. Accordingly, species exist whether we can recognize them or not, and they are not mere imaginary or hypothetical constructs or mere concepts to serve our subjective understanding of nature. I strongly feel that we should indeed stick to perceiving species as naturally distinct units, that is, as objective realities, not just as abstractions.

Another fundamental discussion rests with the question of whether species represent "classes" or "individuals" (see Hennig 1950, 1966, Mayr 1963, 1982, 1988a, 2001, Löther 1972, 1991, Griffiths 1974, Ghiselin 1966, 1975, 1988, 1997, Hull 1976, Mishler and Donoghue 1982, Willmann 1985, Caplan and Bock 1988, de Queiroz and Donoghue 1988, de Queiroz 1995, Baum 1998, Mahner 1998, Bock 2000, Gould 2002). The typological and nominalistic approach considering species as classes ("natural kinds"), as constant types that are separated from any other species by an unbridgeable gap, is to be regarded singularly unsuited to evolutionary and population biology, "where one finds not classes but aggregates of unique individuals, that is, populations" (Mayr 1997: xii) (for an account on the history of the development see also Mayr 1982). Although this particular aspect of species should be of relevance for systematists, for the sake of brevity here the reader is referred to an in-depth discussion of the species-as-individuals theory to reviews and literature therein provided by Rosenberg (1985), Mayr (1988a), and Ghiselin (1997), with a clarification of the metaphysical foundation attempted most recently by Bock (2000).

Clearly the most fundamental, albeit often neglected distinction in this context is between (1) the definition and concept of a species (or German *Artbegriff*) and (2) the species taxon (that is, natural object, or particular) ranked as a category in the Linnaean hierarchy, referring to the central but often overlooked aspect of "conceptualization" versus "categorization." This distinction between the two meanings of the word "species" has explicitly been pointed out by Hull (1965, 2002) and subsequently discussed in Mayr (1982, 1988a, 1996, 2001), Willmann (1985), Frost and Hillis (1990), Bock (1995), and most recently Hey (2001a, 2001b), but see Mishler and Donoghue (1982) and McKitrick and Zink (1988).

Although often confused, species concepts (the meaning of species in nature) and species taxa (as zoological objects to be categorized in an ordering system) are completely different things. Indeed, it is important to distinguish species as a theoretical notion (i.e. concept) from the species category within taxonomy to which species taxa are assigned. Although the idea is that species taxa are unique and fundamental and that the species is also a category within the Linnean hierarchy, we have to be aware that, in contrast, above the species level all other higher categories are not objectively defined, but practical constructs for the purpose of ordering and classifying groups in nature. This categorization as a purely taxonomic procedure is often confused with the conceptualization, that is, the theoretical idea of what species actually are. Being particulars, only species taxa can be described and delimited against other species taxa.

Thus, as Mayr (1996) pointed out, there are actually two different sets of species problems, one being the problem of how to define the species (which species concept to adopt) and the other being how to apply this concept in the demarcation of species taxa. Accordingly, although we describe and recognize species taxonomically, this is only the best approximation of a "real species" we can get. We can only hope and strive to accomplish that our category "species" match up with the real evolutionary groups that are localized in space and time. As naturalists we discover species. We do not create them, however, because they are already there.

In this context, the inconsistent and often confusing way in which the term and concept of species are used in many malacological studies is disturbing. Most taxonomic revisions, for example, do not explicitly or implicitly state the author's concept of species, leaving it to the reader to speculate on the theoretical background from which the author is approaching the case. Other studies at least mention the common problem of species delineation, but define terms such as "species," "genetic species," and even "superspecies" in unconventional ways, inconsistent with any other definition generally used. One example to illustrate this is Ponder *et al.* (1994: 569) in which species are "defined" as "the outgroup and the two ingroup taxa restricted to their type locality," while genetic species are defined as "non-interbreeding genetically definable taxa not readily recognized using morphological data." Although particularly in malacology representatives from individual populations can often be discriminated using shell or other morphological characters, the existence of distinct biospecies remains unclear, as does the conceptual basis used by the researchers.

The choice of which species concept to apply is fundamental to systematics and malacozoology, since phylogenetic, ecological, and other studies are only as good as their underlying data and assumptions. The species concept and the agreement whether species are "real" also have a serious impact on the way we organize collections, view the world, and talk about biodiversity and conservation. For example, any discussions of species richness, any inventory of the fauna, will be seriously dependent on the outcome of this debate. Only if we accept species as real will speciation be a real process and a meaningful problem. Consequently, we need to study the role of reproductive isolation in speciation and look for the geographical context in which species exist.

FROM STAMP-COLLECTING TO EVOLUTIONARY BIOLOGY

Because a species is apparently much easier to recognize than to define, the crucial questions remain: What constitutes species and how do we delimit them? Paradoxically, in systematic practice the most widely used methods are still essentially morphological, i.e. species are treated as phenotypic (and/or genotypic!) units instead of real genetically cohesive communities. As long as naturalists assumed that species were constant with fixed limits and were consistently regarded as separate and independent creations (as was done in and long after Linnean times), this substantially typological approach did not cause theoretical problems.

However, the crucial question of variation increasingly gained attention among naturalists during the second half of the 19th century. With the general acceptance of the Darwin-Wallace theory of evolution, in particular in its modified version of the modern synthesis (reviewed in Mayr 1982, 1991), geographical variation became a central element for the understanding of evolution and speciation, eventually resulting in the increased recognition of the biological species concept (BSC). Under the BSC species were no longer considered as artificial sets defined by phenetic attributes, but as real genetic units, in theory, two taxa form separate species if they are reproductively isolated from one another and incapable of exchanging genes. With the tenet of species being populations of interbreeding organisms, many zoologists started to think about species as being localized in space and time, resulting in numerous studies that paid much attention to phenotypical (and most recently also genotypical) variation in the geographical context.

Research and observation *in situ*, the "geographical principle" that was established explicitly by Alfred Russel Wallace (thus dubbed "Wallace's program" in Glaubrecht 2002) provided the geographical key for the study of the spatial pattern of the distribution of animals as well as for understanding the origin of species and the mechanisms of speciation. It is these geographical data that facilitated insights into complex phenomena in evolutionary biology such as natural selection, faunal regions and their delineation, endemisms and radiations, *formenkreise* and superspecies, as well as the principle of peripheral isolates and the concept of allopatric speciation. Accordingly, providing the knowledge on geographical occurrences of faunal and floral elements over vast areas of the globe in concert with their geographically related variation has to be considered the main contribution of travelling naturalists from Darwin and Wallace to Stresemann, Rensch, and Mayr, who later became instrumental in the development of the modern synthetic theory of evolution (Glaubrecht 2002).

It was the naturalist-explorers' demonstration that not only do individuals exist in nature but directly intergradate and vary geographically within populations. The discovery and documentation of the existence of discontinuities in the natural interpopulational variation of morphological and other markers eventually led to the awareness of the importance of the long-neglected geographical factor, not only for variation and species delimitation, but for speciation and evolution in general. As mentioned above, an early exception to this common neglect during the 19th century was Moritz Wagner (1868, 1889), who pointed out the importance of geography for speciation. Later, Bernhard Rensch (1929) and Ernst Mayr realized how crucial the presentation of a massive documentation in favor of geographical speciation would be, given the then prevailing ignorance of the role of geography in zoology (Glaubrecht 2002).

Recently, molecular genetics has brought some astounding improvements and many new insights into the biological nature of species, but still no resolutions to the species problem. Nevertheless, it is the spatial aspect and the relations along contact zones in concert with microgeographical differentiation that are most promising for contributing new facts towards a solution of the species question.

THE BIOLOGICAL SPECIES CONCEPT

Definition and historical development

The concept of biological species is based on the observation of 19th century naturalist-explorers that populations vary geographically to different degrees and that populations of different species at a given locality coexist but do not interbreed with each other. The most valuable working definition of a biological species is that of Ernst Mayr: "Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr 1942: 120).

Mayr was significantly influenced by a group of ornithologists at the Berlin Natural History Museum, especially the curators Erwin Stresemann [1889-1972] and Bernhard Rensch [1900-1990]. This "Berlin circle" followed a research tradition of ornithologists started by Henry Seebohm [1832-1895] and Ernst Hartert [1859-1933] in England (Haffer 1991, 1992, 1995a, 1997a, 1997b, 1999, 2001, Haffer *et al.*

2000). After gaining many insights from his field experience in New Guinea and the Solomon Islands in 1928-1930, Mayr followed the Berlin tradition of delineating species in a geographical context in his works of the 1930s and 1940s.

In addition, Mayr (1942, 1963) proposed that new sister taxa arise when an ancestral species is subdivided into geographically separate populations that subsequently evolve independently. According to the much favored allopatric model of speciation, the separated forms may accumulate many genetic differences and isolating mechanisms are acquired by incipient species over time so they no longer interbreed when they subsequently meet again (Fig. 1). Thus, new species are not the result of ad hoc selection but of a change of function of properties acquired during the preceding isolation.

As a consequence of Mayr's (1942, 1963) synthetic works, the criterion of reproductive isolation, including the importance of geographical variation, separation (i.e. geographical isolation), and the non-applicability of the degree of (phenotypic) distinctness, was widely accepted as most valuable for defining biological species. The BSC was long considered as best corresponding to the organic discontinuities observed among organisms living in one area. Linking zoological systematics with population genetics, Mayr's BSC became not only a key element of the modern synthesis of evolutionary theory, but was later adopted as the official species definition in conservation legislation, such as the US Endangered Species Act. Using this biological definition of species as interbreeding groups provides an objective and non-arbitrary procedure for determining species status. Reproductive isolation not only represents the only objective criterion available to date, but the evolutionary most meaningful one, and should, thus, be considered the ultimate decisive element in case of conflicting evidence (Mayr, 2000).

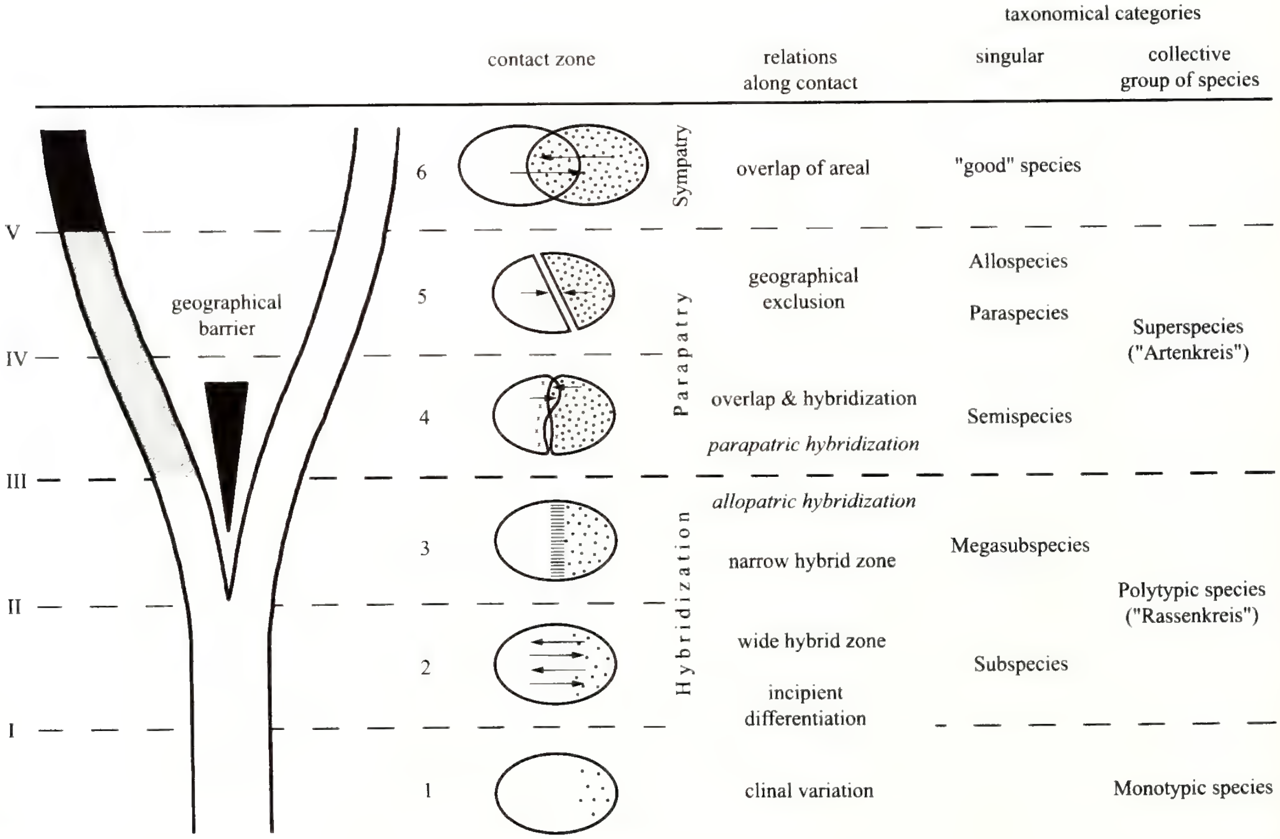
taxonomical categories
contact zone
relations along contact
singular
collective group of species
V
IV
III
II
I
geographical barrier
6
5
4
3
2
1
Sympatry
Parapatry
Hybridization
overlap of areal
"good" species
geographical exclusion
Allospecies
Paraspecies
Superspecies ("Artenkreis")
overlap & hybridization
parapatric hybridization
Semispecies
allopatric hybridization
narrow hybrid zone
Megasubspecies
Polytypic species ("Rassenkreis")
wide hybrid zone
Subspecies
incipient differentiation
clinal variation
Monotypic species

Figure 1. Classifying borderline cases of the microtaxonomic differentiation between subspecies and "good" species. The terminological framework of components in the speciation process is given (under the BSC) as discussed in the text. Stages 1-6 are intermediates in the continuous differentiation of groups of populations. Roman numerals mark the different levels of species limits according to the relevant species concepts, with I-II indicating "cladistic" species categories: I—phylogenetic species, II—evolutionary species, III—biological ("multi-dimensional") species, IV—species under the recognition concept, V—zoogeographical species [Adopted and combined from Haffer (1985: 53, fig. 1) and Haffer (1992: 116, tab. 1)].

Criticism

Even in view of the fact that interbreeding in nature is the finest possible evidence for evolutionary units, the BSC was often held to be non-universal, non-operational and non-applicable (for example Cracraft 1983, Endler 1989, Hull 1997, Mayden 1997, see also more recent discussions in Eck 1998, Wheeler and Meier 2000, Hey, 2001a, 2001b). The BSC was also accused of confusing pattern and process with a bias towards a particular type of speciation. Accordingly, Mallet (1997) criticized that "by postulating an ideal species. rather than a practical approach to sorting actual taxa, Mayr opened a Pandora's box." It was even claimed that the BSC althogether "is not very useful" (Schilthuizen 2001: 19). However, difficulties in the application of the BSC (for example those arising from allopatry, lack of information or cases of incomplete reproductive isolation and hybridization) in themselves do not detract from the validity of the concept. Another cause for misunderstanding the value of the BSC is the lack of distinction between species concept and species taxon (or species category), as discussed above.

Admittedly, there are limitations and a genuine inapplicability of the BSC in the cases of asexuality (uniparental reproduction) and allopatry. Indeed, the BSC is only applicable to organisms reproducing bisexually. For those organisms, however, that reproduce non-bisexually, either completely unisexually (parthenogenesis) or asexually, the agamospecies becomes available, rendering the claim of only a single universal species concept inappropriate. For a discussion of the species concept in parthenogenetic taxa see Maslin (1968), Sudhaus (1984), and Häuser (1987). The existence of reproductive isolation in nature can only be determined with certainty when taxa are sympatric. Given the gradual process of speciation, non-continuous populations may or may not have reached the level of biological species. The status of such taxa, including subspecies belonging to the same species or paraspecies, allospecies, and semispecies (Fig. 1), can be determined by inference only (Mayr 1997, 2001). These inferences have to be made within a taxon-specific framework using the degree of morphological and other differences or, with recent advances in molecular methods, by comparison of genetic distance. Making those inferences is advocated here as necessary and logical procedure that follows from the theory of the BSC. Although McKitrick and Zink (1988: 3) rightly called the inapplicability of the BSC to allopatric forms "an underemphasized problem because there are thousands of allopatric populations," the inability to treat allopatric populations objectively and provide a foolproof system for the correct assignment of isolated populations or other cases of evolutionary intermediacy is inherent in all other species concepts as well.

The BSC is most often criticized as being non-dimensional. However, by viewing a species as existing and extending its populations in a geographical framework, the BSC actually is two-dimensional. With this horizontal notion a biological species appears as a real unit in nature. Although the BSC is admittedly not vertical, that is not primarily a historical concept, if we add the time dimension, three-dimensionality is gained, as suggested by the chronospecies concept. This latter concept (artificial delimiting of portions of phylogenetic species lineages), however, is essentially nothing more than a morphospecies concept applied to fossils. Accordingly, the chronospecies concept uses the criteria of the BSC combined with the morphological approach in geological time (Reif 1984, Willmann 1985, see also papers in Eck 1998).

Expanding the concept

Emphasizing again the distinction between species concepts and species taxa (as discussed above), we find that the species concept is based on the non-dimensional situation, while the species taxon is multi-dimensional. Adding the dimensions of geography and time permits a way to treat populations taxonomically. Applying the ideas of the BSC and, in addition, subsequently testing how many populations and presumed subspecies (due to their distinctness) actually deserve species status even in allopatry, this procedure became a valuable and more heuristic endeavor, in ornithology, for example, than the often meaningless dispute over naming "species" or "subspecies" in allopatric and/or parapatric situations. The last decades have seen the development of the methodological tools to treat the various instances of evolutionary intermediacy. To accommodate taxonomically these stages of the microgeographical differentiation process in concert with the BSC, a terminological framework has been developed, in particular in ornithology (Fig. 1). Terms such as para-, allo-, semi-, and superspecies proved most valuable in the context of zoogeographic and phylogenetic studies, and entire faunas have been studied utilizing this approach (see Haffer 1985, 1992, Amadon and Short 1992, Sibley and Monroe 1990, see also literature cited therein for examples from ornithology). Adequate systematic inferences and the application of allo-, semi-, and synspecies of a superspecies is advocated, for example, by Mayr (1942, 1963), Amadon (1966), Sudhaus (1984), Haffer (1986), Sibley and Monroe (1990), Mayr and Ashlock (1991), Glaubrecht (1993, 1996, 2000), and Helbig (2000).

THE DEBATE CONTINUES

How to delimit species in a cladist's world?

The last two decades have seen a particularly active phase of debate about how to define and delineate species in a cladist's world. Phylogenetic systematics, as proposed originally by Hennig (1950, English translation 1966, see also Ax 1984 and Wägele 2000), and particularly its application and recent computation utilizing advanced methods in bioinformatics and technology by cladists, produced a revolution in systematics. Unquestionably, the rigorous application of cladistic analysis had a major impact also on many other aspects of systematic biology, resulting in "tree thinking" (O'Hara 1994). Because the BSC was considered insufficient for this purpose, phylogenetic thinking, or the cladistic approach to nature, necessitated the re-evaluation of concepts in systematics, with cladistics recognizing the importance of a species concept that serves their methodology of branching patterns and clades defined by synapomorphies, leading to "(re) inventional word games," as Avise (2000b: 1831) rightly noted in his elegant and eloquent book review on "the speciational wonderland." Currently, there are 22 ways to view and perceive what a species is, according to a review by Mayden (1997); Hey (2001b) lists 24 concepts. While the battle over the best cladistic species concept continues among cladists, proponents of the BSC, especially Mayr (2001), denied that any of the new phylogenetic concepts is legitimate, since "none of the authors of these new concepts has understood the difference between a species concept and a species taxon. Instead of new concepts, they have proposed new operational criteria of how to delimit species taxa."

Following a suggestion by Haffer (1992, 1998) and Gittenberger (1972), who both tried a systematization among the multitude of species definitions instead of a mere compilation, I will distinguish here between "horizontal" and "historical" species concepts. Although a species as defined by the BSC can be viewed as a horizontal cross-section of a phyletic lineage at any given time, a historical species concept results in the vertical delimitation of species as suggested, for example, under the Hennigian Species Concept (HSC) (Meier and Willmann 2000), the Evolutionary Species Concept (ESC), and (implicitly) under the Phylogenetic Species Concept (PSC). Proponents of the HSC, ESC, and PSC consider species to be parts of a "vertical" evolutionary lineage between two consecutive cladogenetic events (that is speciation and/or termination through extinction). The following brief review will restrict itself to the vertical concepts ESC and PSC, other concepts are considered of minor importance, because they reflect only special aspects with slightly changed emphases.

The Evolutionary Species Concept

Simpson (1961: 153) suggested defining species as "a lineage (an ancestral-descendent sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies." Later, Wiley (1978: 18) proposed in a slightly revised version that "a species is a single lineage of ancestral descendent populations of organisms which maintains its identities from other such lineages and which has its own evolutionary tendencies and historical fate." More recently, Wiley and Mayden (2000) have redefined and defended the evolutionary species as "an entity composed of organisms which maintains its identity from other such entities through time and over space and which has its own independent evolutionary fate and historical tendencies."

Although considered relevant for both living and extinct groups and to sexual and asexual organism, the ESC is nonoperational and subjective (that is, containing undefinable criteria rendering them useless in practice), and even Simpson himself has abandoned his own concept as being rather nebulous for systematic purposes (Reif 1984, O'Hara 1993, Mayr 1992, 2001). How, for example, is one to describe and determine "evolutionary tendencies" or "historical fate" of a population or taxon? Nevertheless, the ESC is still supported and frequently recommended (e.g. Maslin 1968, Wiley 1980, 1981, Ax 1984, Willmann 1985, Otte and Endler 1989, Frost and Hillis 1990, Mayden 1997, Peters 1998). However, the ESC failed to provide its main objective, namely a clear delimitation of a species in the time dimension that turned out to be illusory in all cases of gradual species transformation, as is illustrated in particular by the classical *formenreihen* of freshwater gastropods (see Willmann 1981, Williamson 1981, further discussions in Reif 1984, Eck 1998). In essence, the ESC is a typological morphospecies concept that is not operational; it can only assume that characteristic features are consistent and thus diagnosable throughout the entire historical existence of the evolutionary lineage.

The Phylogenetic Species Concept

Cladistic methodology views the world as branching patterns. According to this philosophy, every lineage starts and ends with a branching event (speciation) or its extinction and is characterized by at least one autapomorphy. This view led to the need for a species concept consistent with phylogenetic principles. The reproductive criterion (i.e. breeding compatibility) is considered inappropriate among cladists to group organisms into species. Instead, the existence of unique patterns of shared and diagnosable characters is proposed and defended as a sufficient criterion.

Espousing PSC as alternative to the BSC, for example, Cracraft (1983: 170) defined species "as the smallest diagnosable cluster of individual organisms within which there is a parent pattern of ancestry and descent." He later defined species under the PSC as "an irreducible (basal) cluster of organisms diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent" (Cracraft 1989: 34-35). For a historical review and application of the many versions of the PSC see Mishler and

Brandon (1987), McKitrick and Zink (1988), Mayden (1997) and Mishler and Theriot (2000).

One of the most serious problems with the PSC is that there are too many versions, which leads to confusion about the specific definition. Regrettably, irrespective of the two decades of debate, cladists have failed to set or agree on any standards for what to consider a "phylogenetic species." This lack of consenus is both a problem for communication and acceptance, and is a source of much confusion and many misconceptions, which hampers scientific progress. Mayden (1997) sorted out two main approaches, the diagnosable version and the monophyly version of the PSC, the latter stemming from the debate of cladists whether the concept of monophyly should be extended from higher categories to the species level (de Quieroz and Donoghue 1988, Nixon and Wheeler 1990).

The diagnosable version

In the definition of Nixon and Wheeler (1990: 218) a species is "the smallest aggregation of populations (sexual) or lineages (asexual), diagnosable by a unique combination of character states in comparable individuals (semaphoronts)." Because this definition is character-based it renders a phylogenetic species analogous to a morphospecies, even if now allowing to do so on an additional level, such as the molecular features. Accordingly, using the increasingly improved methods of molecular genetics, it currently becomes more and more standard procedure to characterize each and all geographically separated populations by apomorphic features (i.e. sequence differences), with smaller populations more easy to track than large polymorphic ones. In conclusion, those populations which turn out to be consistently diagnosable by characters or character combinations recognizable by ordinary (= arbitrary) morphological means and/or molecular means (single fixed nucleotide base pairs) are considered as species under the PSC.

The monophyly version

Rosen (1978) stated that "a geographically constrained group of individuals with some unique apomorphous characters, is the unit of evolutionary significance." More recently, Mishler and Theriot (2000) defined species as "the least inclusive taxon recognized in a formal phylogenetic classification." Accordingly, "taxa are ranked as species. because they are the smallest monophyletic groups deemed worthy of formal recognition." This concept equates species with monophyletic units and speciation with character transformation. There are a few problems with monophyly in this context, for example, the question of whether species are, must, or can be monophyletic (Wiley 1981, Willmann 1983, McKitrick and Zink 1988, Wheeler and Nixon 1990). Establishing "monophyly" for species has proven difficult in practice, as Nelson (1989) admitted.

Generally, PSCs have been criticized for three different reasons. In addition to being (1) typological and to diagnose evolutionary units on the basis of (partly trivial) characters, they are (2) arbitrary and reductionistic in the sense that they not include important biological criteria, and are (3) leading to the recognition of too many species. Apart from the question of what actually is "phylogenetic" about a species, for example, Mayr (2001: 167) criticized the various phylogenetic species concepts as "simply typological prescriptions of how to delimit species taxa." Starting from the conviction that without clear-cut definitions no progress in the clarification of concepts and theories is possible, it is not helpful that in the last two decades so many cladists have come up with several more or less divergent definitions of the phylogenetic species concept. Often even the same authors have different formulations and wordings at different times, without reaching any compelling synthetic consenus. This results in confusion of what an author means when referring to a species under the PSC and it certainly defeats the cladists' claim to provide with the PSC a viable alternative to the BSC. Given the purported demise of the BSC, the recent debate, illustrated in Wheeler and Meier (2000), reveals that the "revolutionaries," as Avise (2000b) stated, have not come up with something much better.

In addition to the often rather nebulous and vague usage of words in the PSC definitions, any focus on diagnosable differences between phylogenetic lineages, not necessarily representing populations, renders it reductionistic and non-biological, because it ignores the relationship to other populations or taxa within a geographical and historical context. The operational orientation of the PSC (serving better diagnosability) not only leads to subjectivity, it also renders the PSC a clearly typological concept close to essentialism, which had been overcome with the modern synthetic theory of evolution. As a result, the PSCs will eventually lead to the same ballooning of species numbers as observed in the 19th century.

In combination with the arbitrariness of changing delimitation of species under the different versions currently available, the PSCs render comparative studies of faunas and speciation processes hazardous. Only very disputably is the PSC truely a species concept, i.e. in the sense of having any relevance to a species as a natural entity in nature. Defined in this way, a phylogenetic species does not play any role in the ecosystem nor seem to have any interaction with other populations of the same species or with other species, but only serves as a description of a taxon on a cladogram. In addition, the various stages of differentiation in geographically vicariant populations or taxa are not distinguished taxonomically.

Because of the subjectivity of delineating populations in the patchy, allopatric situations of continental areas, the application of the PSC under the diagnosability version endangers consensus among systematists and, therefore, taxonomic stability (see critique in Eck 1998, also Snow 1997, Haffer 1995b, 1998, Wheeler and Meier 2000). Irrespective of these problems, the PSC has been widely stated as providing an objective species concept. It was strongly advocated, first by ornithologists (for example see Cracraft 1983, 1989, McKitrick and Zink 1988), but later also by other practical zoologists (see Kottelat 1997 and Lydeard *et al.* 2000 for two examples).

Implications and consequences

Proponents have failed to develop a single useful, standard definition for a "phylogenetic species" that secures congruent use. In addition, the PSC is an attempt to combine two widely disparate concepts, namely monophyly and diagnosibility, into one species definition, which increases the number of practical and theoretical problems (see for example Sluys 1991). Thus, with respect to the alternative definitions under the PSC, the criteria given do not achieve the demanded degree of objectivity. Ultimately, the immanent subjectivity of the definition will result in arbitrary species delimitations.

In contrast, the criterion of reproductive isolation under the BSC provides an objective means of separating sympatric species. This criterion also represents the causal factor that produces and maintains discrete entities. Avoiding an inflation of "species" by naming even slightly differentiated forms or populations with unbalanced degrees of differentiation, which can happen using the PSCs, the BSC makes an attempt to delineate taxa as species with respect to the same degree of differentiation.

When systematists apply a narrow morphological species concept, they arrive at higher numbers of species. For an example from birds see Figure 2, for other examples from fishes and insects that apply the PSC see Kottelat (1997) and Packer and Taylor (1997), respectively. Thus, so-called "wide" versus "narrow" approaches of delineating species have direct consequences for the assessment of biodiversity and conservation. As a consequence of the perception of species as diagnosable units, the application of PSCs will result in a great proliferation of species and inflate the biological diversity on the lowest taxonomical level, even if one accepts only phenotypic differences and not molecular genetic differences.

In conclusion, the PSC is certainly not an improvement. Although not perfect, the BSC is still the most useful and meaningful concept, while PSC lacks objectivity and is a step backward to the days of typological thought. Not by accident, the BSC became the "working definition" of species among most population and evolutionary biologists for most of the last century, although it was formulated not for convenience but for its correspondence to natural phenomena, as Coyne *et al.* (1988) pointed out. Taken together, the problems with the BSC are fewer than those faced by other species concepts, particularly those based on morphology. Therefore, the difficulties of applying the BSC are not such as to justify its rejection in favor of other, logically and biologically worse concepts. The BSC is to be favored because it is the only definition that is based primarily on the biological significance of a species.

TWO EXAMPLES FROM FRESHWATER GASTROPODS

Initially in malacology (when it was perceived essentially as conchology), a plethora of nominal species were described, followed by only a slight tendency to reduce the numbers of species after population thinking was implemented in some areas of the study of molluscs. Generally, it was concluded that much of the observed conchological variation actually represented population-level phenomena. However, in the absence of studies explicitly focussing on reproductive isolation in sympatry, most taxonomic decisions are still largely subjective and primarily based on morphology.

Because more freshwater biotopes occur in isolated areas than do marine or terrestrial ones, populations of freshwater gastropods tend to be isolated (Rensch 1929, Hubendick 1954, Meier-Brook 1993, Glaubrecht 1996). This discontinuous distribution not only leads to morphological variations in isolated populations and microgeographic races, but consequently also results in the naming of nearly each of these populations as distinct species under typological-morphological species concepts. This will also be the case under the PSC, because these concepts do not take into account biological phenomena such as geographical distribution and genetic cohesiveness, even of temporarily separated populations.

Two case studies of freshwater gastropods of the former "melaniid species basket" ("Melaniidae" = Thiaridae *sensu lato* of the superfamily Cerithioidea, see Glaubrecht 1996, 1999) illustrate this point, using taxa of *Lavigeria* Bourguignat, 1888 from Lake Tanganyika (which should be grouped as belonging to the Paludomidae instead of Thiaridae *s. str.*, see Glaubrecht 1999, Strong and Glaubrecht 2002), and the North American Pleuroceridae.

Case 1: "Le Bourguignatisme"—an example from Lake Tanganyika

One of the most unhappy episodes in the history of malacology is the French school of the so-called "Nouvelle

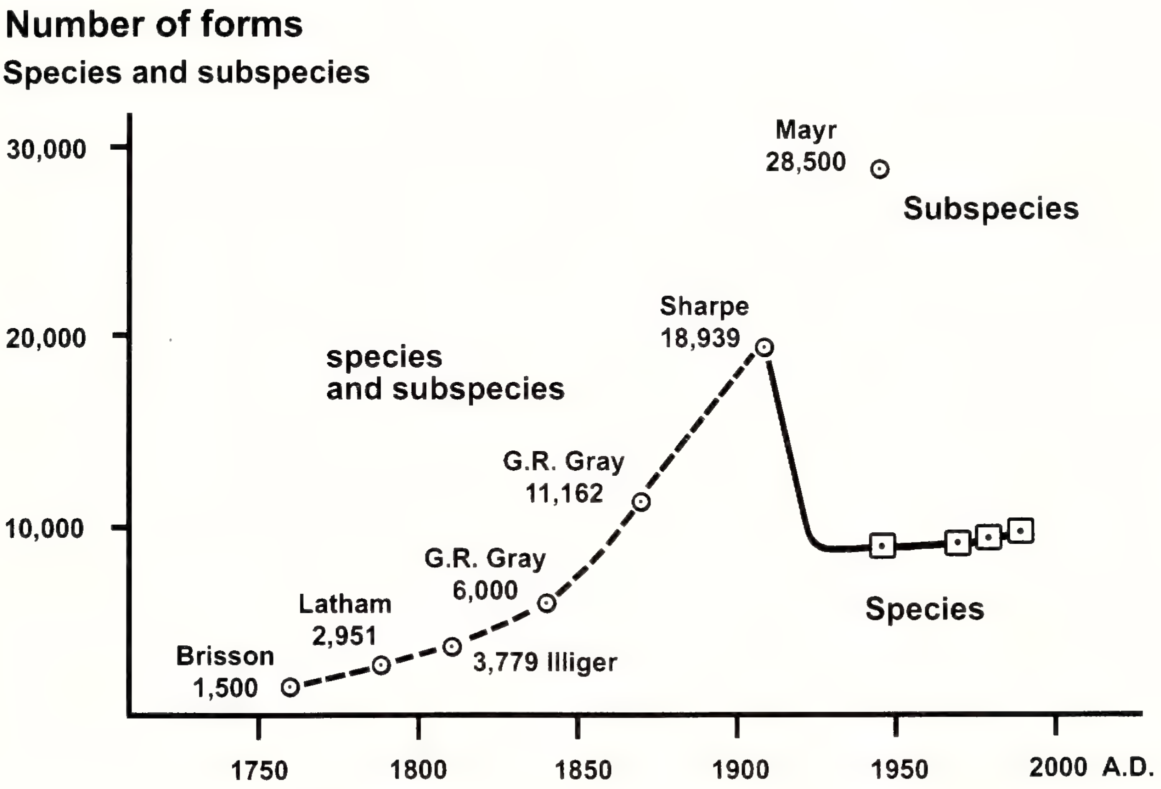

Figure 2. Implication of applying different species concepts. Marked by the works of ornithologists during the last 250 years the increase of numbers of species and subspecies of birds is shown, culminating in the recognition of 18.939 species around the turn to the 20th century. Applying the multidimensional species concept (under the theory of the BSC and influenced by the "Berlin circle" and Ernst Mayr, see text), eventually halted this process after 1900. Reversing the situation during the 1920s and 1930, many morphospecies were reinterpreted as subspecies and combined in more widely conceived biological species taxa. Immediately, this resulted in a precipitous decline in the number of species recognized. It was the emphasis of the existence of closely related allopatric and parapatric species (together forming a superspecies) that led to a moderate stability regarding species numbers during the 1930s and 1940s, and to an estimated total number of known birds of today around 9700 species. This process, starting during the late 1920s, when geographically representative biospecies were discovered, can be aptly called a "quiet revolution" in systematics, that is still missing in malacology [after Haffer 1992: 147, fig. 4].

École," with Jules-René Bourguignat [1829-1892] as its main proponent. Bourguignat considered as distinct species (and merited a name to) taxa that could be distinguished on the grounds of three or more constant characters. Called the "*bête noir* of European malacology" (Dance 1970) and the "great species manufacturer" (Kobelt 1881), Bourguignat was the most radical among conchological splitters. Unable to comprehend or accept the concept of species as a biological entity, he regarded species as abstractions that did not exist outside his imagination.

One of Bourguignat's special interests was the so-called thalassoid (marine-like) molluscan fauna of Lake Tanganyika. Someone who could conjure up dozens of novelties from an average European lake or make 20 species out of a well-known species of European freshwater mussel, could work miracles with these thalassoid gastropods. Based on collections from various French naturalists, Bourguignat described 75 new "species" and proposed 9 new genera in his final masterpiece, the "Histoire malacologique du Lac Tanganika" (Bourguignat, 1890). For example, he split *Lavigeria* as we know it today into five genera and compiled 51 named species for it, introducing 46 new species between 1885 and 1890 (another 7 species were described in subsequent decades). In contrast, only one or two species, *Lavigeria nassa* (Woodward, 1859) and *Lavigeria grandis* (Smith, 1881), respectively, were accepted by Leloup (1953) and Brown (1980, 1994) under the (implicit) application of the BSC. For an illustration of the taxonomic history of *Lavigera* see Table 1.

Bourguignat and his colleagues can be excused by the fact that they lived when there was little agreement over the species problem. However, this situation is quite different from today with some systematists only being dissatisfied

Table 1. The taxonomic history of *Lavigera*, a thalassoid gastropod endemic to Lake Tanganyika, East Africa, illustrates the changing number of species (and generic) names applied under different taxonomic concepts.

Author	No. of species	No. of genera
Bourguignat (1890)	51 species	5 genera
Pilsbry and Bequaert (1927)	22 species	1 genus
Martens (1897)	10 species	1 genus
Leloup (1953)	2 species	1 genus
Brown (1980, 1994)	2 species	1 genus
Michel (2000)	20+ species	1 genus
Todd and Michel (2001)	30+ species	2 genera

with the answers already available to the species question. It is interesting to note that this dissatisfaction has again resulted in an increase of the number of named species in *Lavigeria*. While only two species were accepted since Leloup's (1953) treatment, more recently the existing morphological disparity in Lake Tanganyika has been approached by naming the smallest diagnosible units (as suggested, for example, under the PSC) and implying a local radiation within this genus (e.g. Michel 2000, Todd and Michel 2001). Regrettably, not only is Bourguignat's typological approach repeated this way, but also a general weakness of his treatment, i.e. proposing high numbers of species in the absence of providing a modern systematic revision.

Thus, it is currently difficult to understand the biology and systematics of the genus *Lavigeria*. For example, it has been proposed that viviparity in Lake Tanganyika gastropods in general and in *Lavigeria* in particular was a major factor in the causation of the radiation of species flocks and species richness, respectively (Cohen and Johnston 1987, Michel 1994). This claim has been discussed and rejected by Glaubrecht (1996, 2001, see also Strong and Glaubrecht 2002). First, most thalassoid gastropods are actually not viviparous but oviparous, and second, those genera that are viviparous, other than *Lavigeria*, in particular *Tanganyicia* Crosse, 1881 and *Tiphobia* Smith 1880, are monotypic. What is special about *Lavigeria*? This taxon has a unique morphological diversity, tempting Todd and Michel (2001) to, "delimit working species-concepts using these shell characters, independent of geographical considerations to prevent occurrence information biasing our identifications and then assign the nominal species to our concepts." Apart from the authors' unconventional idea of what a "concept" of a species is and how to "assign" the latter to the former (see discussion above), this procedure resulted in their conclusion that their "systematic framework for the genus currently consists of over 30 species," and that "many more species remain to be discovered as sampling improves" (Todd and Michel 2001: 355). The question remains unanswered what natural (speciation) mechanism causes this enormous species flock to evolve and whether there is any ecological and/or geographical correlation indicative of, for example, habitat specificity and fragmentation and/or intralacustrine allopatry. In contrast to the procedure chosen by these authors, who do not want to be "biased" by information on occurrences, evaluating the actually morphological disparity and purported taxonomic diversity in *Lavigeria* within a microgeographical framework that includes the ecological context would be a most promising research program to address the species question.

Case 2: The species question in North American Pleuroceridae

The Pleuroceridae has long been recognized "as one of the most difficult families of American mollusks," (Pilsbry and Rhoads, 1896: 495). For more than a century, high degrees of shell variation caused authors to describe a plethora of species and subspecies. The bewildering variety of shell phenotypes, interpreted as the result of an extensive endemic radiation particularly in streams and rivers of the southeastern USA posed tremendous problems to the systematics of this group. Accordingly, Dillon (1984: 70) noted that "pleurocerid taxonomy is currently in a confused state."

The outstanding (and still most comprehensive) systematic monograph of this family by George W. Tryon (1873) listed a total of 464 species for North America. In his treatment of the genus *Elimia* H. and A. Adams, 1854 (= *Goniobasis* Lea, 1862) alone, Tryon recognized 255 species. He later clearly saw that a reduction of pleurocerid species must be made, coming to the belief in 1888 that "there were not more than a tenth as many good species as names" (see Pilsbry and Rhoads 1896: 496). In the introduction to his monograph, Tyron (1873: li) had remarked concerning the morphological variation found in pleurocerids: "We thus find that no one character (with very few exceptions) can be relied on in species discrimination, but rather a combination of characters, with a general idea of the necessary allowance for variation pervading other species of the same general type, or contiguous locality."

Like many of his contemporaries, Tryon was aware of the species problem but not of the solution to it. In adding to his monograph the correspondence with another contemporary malacologist, James Lewis, Tryon gave some insight into the debate. For example, in discussing the most variable species from the creeks in Tennessee with "a perfect series of differentiations of carinated apices," Lewis (cited in Tryon 1873: 424-426) remarked that "one cannot tell where to assign limits. Limits are apparently obliterated and species have no existence. We are very largely at the mercy of opinion, some of which, no doubt, are but the reflex of the idiosyncrasies of the persons with whom they originate."

What Lewis very aptly called the "key to the origin of many of our species" provides an explanation for the typological species-making in freshwater gastropods. Because it was common practice of local collectors to send only single shells for identifications to experts of the group, it appears as no "wonder then, that the descriptive naturalist should unwittingly fall into a very natural mistake and describe these shells as new species" (see Tryon 1873: 426)

Pilsbry (*in* Pilsbry and Rhoads 1896: 496) was aware that the same species often occurs in some localities with the shell sculptured throughout, in others with sculpture only on the upper portion, and in still other localities only with the characteristic sculpture on the earlier whorls. Anticipating a research program finally taken up much later, he concluded that "these shells must be collected and studied by river-systems." Goodrich (1940, 1942), for example, studied members of the Pleuroceridae in the Ohio river system and the Atlantic coastal plain, compiling data for 81 species from these drainage complexes (leaving others unmentioned, however). Thus, he first tried to sort out named shells from real biological entities and to clarify some of the confusion over the various names erected for this over-described family of aquatic snails.

In his compilation of North American freshwater snails, Burch (1982) provided the most and only recent overview, listing a total of 212 pleurocerid taxa (including subspecies and "morphs"), of which 152 were attributed species status. For the diverse and morphologically disparate genus *Elimia* alone he reduced the number given by Tryon (1873) by two-thirds, recognizing 83 species. Although it is generally realized now that there are problems with the delineation of species based solely on shell morphology in snails that exhibit clinal variation, Burch's compilation still provides the only attempt so far to comprehensively treat the entire group. Nevertheless, any attempt to revise the Pleuroceridae and provide a formal systematic monograph is lacking, probably due to the enormous problems caused by the chaotic taxonomy resulting from former typological approaches.

Several studies comparing the amount of phenotypic and genotypic variability in pleurocerid species from various river drainages, using measurements of genetic divergence/similarity based on allozymes (for example Chambers 1980, Dillon and Davis 1980, Dillon 1984, Dillon and Lydeard 1998) or mitochondrial sequence data (Lydeard *et al.* 1997, 1998, Holznagel and Lydeard 2000, Mihalcik and Thompson 2002), reveal conflicting evidence as to the morphological and genetic/molecular concordance within and among species and genera of pleurocerids. From the results on pleurocerids it was concluded, (1) that morphological variability is correlated with environmental differences, (2) that species identification using shell morphology alone is often unreliable, (3) that because intrapopulation genetic variation is low and interpopulation divergence is high gene flow even among conspecific populations connected through water can be quite low, and (4) that there are different views of species relationships and taxonomy based on electrophoretic studies and molecular genetic data compared to previous work based on shell morphology. For example, for taxa of the *Elimia* (= *Goniobasis*) *floridensis* (Reeve, 1860) species complex in Florida, Chambers (1980) reported that the divergence in shell sculpture was accompanied by little or no genetic divergence, which has been greatly facilitated by the low frequency of dispersal between drainage systems. Given that the geographic distribution of these freshwater gastropods are subdivided by the discontinuities of their habitat, Chambers (1980) favored an allopatric model of speciation when concluding that geographic barriers between populations have probably played a major role in promoting the complex pattern of speciation observed in the evolution of Pleuroceridae.

Studying species of *Elimia* occurring from Virginia to Georgia, Dillon (1984) emphasized a strong correlate of geographic distance with genetic divergence between populations. Thus, although the range of a species is fragmented into a large number of isolated populations separated from one another by mountains between drainages and by stretches of large, apparently uninhabited river (Dillon and Reed 2002), genetic cohesion is maintained even with negligible gene flow. Geographically isolated populations not sharing alleles at many studied allozyme loci did not demonstrate reproductive isolation, as Dillon and Lydeard (1998) noted. Similarly, in a study of the species of the pleurocerid genus *Leptoxis* inhabiting the Mobile River basin of Alabama, Dillon and Lydeard (1998) found some of their data to be more consistent with a hypothesis of geographic isolation rather than reproductive isolation (see also Dillon and Reed 2002). Nevertheless, they strongly advocate special attention and conservation status for those pleurocerid populations to which species status would be attributable on the basis of high genetic divergence.

With respect to the number of species as well as how and where to delineate species-level taxa in Pleuroceridae, many contradicting arguments have been put forward, at least in part based on the considerable mismatch between morphologically distinguishable taxa and those found either by electrophoretic studies or by molecular genetic analysis (mtDNA). For example, stating that certain shell characters (for example, sculpture) can give a misleading view of interspecific boundaries and relationships, Chambers (1990) recognized only four species of *Elimia* in Florida river drainages, namely *Elimia floridensis* (Reeve, 1860), *Elimia dickin-*

soni (Clench and Turner, 1956), *Elimia boykiniana (*Lea, 1840), and *Elimia curvicostata* (Reeve, 1861), where earlier treatments had considered 10 species. In contrast, Thompson and Mihalcik (2002) and Mihalcik and Thompson (2002) identified the previously recognized *Elimia curvicostata* from rivers in western Florida to Georgia, for which Chambers has listed 10 junior synonyms, as a complex of 14 morphologically distinct species, describing five new species and two new subspecies. These authors propose that because of convergence in adult shells, the juvenile shells are of primary importance in distinguishing species. In their parallel molecular analysis they found five distinct species clusters that correlate geographically to different river drainages. Earlier, Thompson (2000) described, based on morphological evidence only, four additional species of *Elimia* from the Coosa River drainage in Alabama.

A similar association of clades in a molecular phylogeny with drainage basin rather than with traditional morphological groupings of the currently recognized taxa was found in studies of the pleurocerids from the Mobile Basin (Sides 2002). Minton (2002) found, in a cladistic analysis of the genus *Lithasia* from the Cumberland, Ohio, and Tennessee River drainages that morphological characters (shells and radulae) alone neither recover currently or historically recognized groups at the species level nor do they match with those taxa delineated based on molecular phylogenetic analysis (see also Lydeard *et al.* 1997). In addition, Minton and Savarese (2002) found evidence for the existence of an undescribed phylogenetic species in the Harpeth River, Tennessee, this time explicitly applying the concept of phylogenetic species in their study.

Based on studies on genetic variation at allozyme loci among populations of two species of *Elimia*, *Elimia proxima* (Say, 1825) and *Elimia catenaria* (Say, 1822) from the Atlantic drainages of the Carolinas, Dillon and Reed (2002) called into question the species' identifications and status of some nominal species and subspecies and their relationship in neighboring Atlantic drainages. For example, they suggested that *E. catenaria* might occur also in Georgia (and maybe even further south), instead of applying different names to populations with slightly distinct morphological (shell) characters whenever found in different drainages of an adjacent state.

Although the century-old suggestion to study pleurocerid systematics by river systems instead of typological naming of individual shells has finally been taken, a general disagreement on how to apply species concepts to these highly polymorphic freshwater gastropods in light of new biochemical methods has not yet greatly improved the situation. Currently, the systematics of Pleuroceridae are constrained between the Scylla of a relatively wide approach of molecular phylogenetics that chiefly resolves intergeneric relationships in an effort to understand the evolution of the entire family (Holznagel and Lydeard 2000) and the Charybdis of a narrow focus on populations within individual rivers or drainages and a restriction to only few species-level taxa (Dillon 1984, Lydeard *et al.* 1997, Dillon and Reed 2002, Minton 2002, Sides 2002). Taking the geographical context into consideration on a larger scale, such as comparing congeneric taxa like *Elimia* or *Leptoxis* across their entire distributional ranges and all inhabited drainage systems, in concert with a cladistic analysis of morphological and molecular data would greatly enhance our understanding of the nature of species in these North American gastropods.

CONCLUSION

> Nature, in some respects, comes to us as continua, not as discrete objects with clear boundaries . . . But since nature has built a continuum, we must encounter ambiguity at the center. Some cases will be impossible to call—as a property of nature, not an imperfection of knowledge (Gould 1985)

Species as dynamic entities

Species are, and therefore should be conceived of as, dynamic entities that need to be placed in historical as well as geographic contexts. Biological discontinuities such as reproductive isolation by which the species are characterized in nature should be utilized to define them. Among the plethora of species concepts suggested in the past, the BSC and the PSC(s) confront us with the twin dangers of either "overlumping" obviously distinct specific variation (on phenotypic and on genetic grounds) via strict application of the BSC, or oversubdividing biodiversity on lowest taxonomic levels. Because things in nature that seem distinct may represent the extremes of a continuum, I have emphasized (1) the historic dimension of the species debate and (2) the horizontal dimensionality of the species concept, that is the geographical factor in the discussion on the nature of species. In order to recognize biological species as evolutionary and ecological units we need to combine data on geographic variation with information on dispersal and environmental history (i.e. the biogeographical patterns). To this purpose, the BSC provides the only non-arbitrary criterion available, namely the presence or absence of interbreeding between two populations coexisting temporally and spatially. In contrast, the PSC determines species status based on the subjective and arbitrary criterion of diagnosability (that is, species as the smallest diagnosable units).

Biologists should be more aware and, consequently, explicit in applying different conceptual approaches to the species problem. If not using the concept of a biological species as reproductive community but focussing on diagnosability only (either at the morphological or molecular level), authors should explain their line of argument as to their perception of species in nature. For the most interesting and spectacular case studies of enlarged species diversity as recently discovered, for example, in limnic hybrobiids in North America and Australia, or some thalassoid molluscs in ancient lakes such as Lake Tanganyika and the central lakes on Sulawesi, the taxonomic descriptions of the many new taxa should be supplemented by addressing the general problems of species discrimination with a non-essentialistic species concept. Within the framework of evolutionary knowledge and population thinking the discussion and analysis of speciation in those cases would certainly enrich the century-old debate on the orgin of species diversity.

There are many approaches in malacology today to overcome the purely descriptive tradition that resulted from the often uncritical multiplication of taxa names during the typological times of the 19th century. Modern taxonomy is increasingly aware of the uniqueness of individuals on the one hand and the wide range of variation within any population of individuals on the other hand. While malacology is often too narrowly focussed on accumulating data, other disciplines, such as ornithology, led the way in testing general evolutionary theories, including the predictions from species concepts and speciation hypotheses. What is needed is the integrated synthesis between the malacologist compiling observations from the field and laboratory and the malacologist evaluating theories within the framework of historical achievements.

Rather than a lack of definitions, the real neglect is the absence of a clear statement of why and on which grounds decisions on species status have been made. Too often in systematic revisions and other taxonomic accounts, any reference to the species concept is either lacking or the definitions given and/or used are unconventional, incorrect, or misleading. As long as this situation continues, the progress in systematic science is hampered as much as during Darwin's days, when "different naturalists made different decisions on different grounds, with the result that the decisions—and the entities dealt with—certainly did appear purely arbitrary" (Kottler 1978: 296). In this context, and in turning around the traditional tendency to look and describe "specific" differences, we should start with a single species as null hypothesis. In examining any set of morphological and genetic data we should only accept the more complex hypothesis of two or more species if a better fit with the data available necessitates this.

Towards a phylogeographical synthesis

There is a long research tradition in zoology of geographical variation and the characterization of geographic varieties. We need to re-vitalize this tradition and at the same time employ newly available molecular and other techniques, as exemplified recently in phylogeography. This field of study is concerned with the principles and processes governing the geographical distribution of genealogical lineages, especially those within and among closely related species (for review see Avise 2000a). A primary requirement of the expansion of empirical studies of comparative phylogeography is the acquisition of biogeographic information on a regional scale. In many cases in invertebrate zoology, however, those basic biogeographic data are not available, thus hampering the integration of genealogical data. Concerning the question of how to delineate species, we are not suffering from a lack of definitions, but rather from incomplete biological information. The recent molecular revolution of phylogenetics with the now widely-used methods of PCR and sequencing has provided powerful tools for species-level studies based on the reconstruction of past events and geographic modes. For example, within limnic molluscs with confusing taxonomy and poorly understood biogeography, this is most recently exemplified by the mudsnail species of the hydrobiid genus *Hydrobia* (Wilke *et al.* 2000) and by the limnic bivalves of the genus *Corbicula* (Pfenniger *et al.* 2002).

There is a great need for the integration of more data, not only on morphological and molecular variation but also on geographic distribution. The fact that the range of intraspecific variation over a given region is often insufficiently known renders any evaluation of gene flow among populations hazardous. The importance of knowing the structure of the population genetics of a species or species complex as a prerequisite for determining the genetic units has been illustrated recently for the epidemiologically important vector of malaria *Anopheles gambiae* s. str. (della Torre *et al.* 2002) and for some snails that are vectors for schistosomiasis (reviewed recently by Blair *et al.* 2001).

Future challenges

Any in-depth debate of the species question in malacology faces two major challenges: first, to get more of the relevant data for as many taxa and case studies as possible and second, because natural processes are constrained by a three-dimensional space, to make inferences in the appropriate spatial and temporal context. To gain the data for these inferences, the various stages of differentiation, particularly in contact zones and nearby areas, should be focussed on, and molecular and morphological variation tested in allopatry, parapatry, and sympatry, with the aim of attributing the status of allospecies, paraspecies, or semispecies to local populations (Fig. 1). Attempts to make these inferences are led by the conviction of Stebbins (1969), albeit in another context, that "the best system for any group is one synthesized from data of all kind." Scrutinizing our ideas on the nature of species thus demands the integration of morphology (from diagnostic biometry to anatomy and histology), molecular genetics, and biogeographical analyses supplemented by data from ecology, ethology, and other sources. Avise (2000a, 2000b) suggested that wedding the better elements of the traditional BSC and PSC will eventually produce a synthetic conceptual framework for species recognition. In this ongoing phylogeographic synthesis, population-demographic and population-genetic principles should be supplemented by historical geographic considerations. Instead of trying to find another species definition or concept, we should make use of the heuristic properties of the existing biological and phylogenetic ones.

A phylogeographic approach that combines the reproductive criterion of the BSC (such as barriers, isolates and geography) with the phylogenetic criterion of the PSC (namely historical and demographic aspects) will eventually lead to a most fruitful synthesis. The increasingly better and more detailed documentation of morphological and molecular genetic differentiation of molluscs in their spatiotemporal context will result in a taxonomically improved classification based on insights from biology and phylogeny. Freeing malacology from the typological naming of whatever was previously diagnosed as "species" will then truly become Leopold von Buch's legacy.

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Are populations of physids from different hot springs distinctive lineages?*

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Abstract: A number of physid species, including *Physa johnsoni* the Banff Springs snail, were first described from hot water environments. Physid populations have a remarkable ability to cope with warm to hot temperatures. We present a comparative genetic analysis of the physid species living in hot springs in the context of a larger preliminary phylogeny of the Physidae. The molecular phylogeny, which was based on partial DNA sequences of the mitochondrial cytochrome oxidase c subunit 1 and 16S rRNA genes, place *P. johnsoni* in the *Physa gyrina* group, indistinguishable from other members of the Western United States within this group. Included in this *P. gyrina* group are the following putative species: *P. johnsoni*, *Physa aurea* (actually of the Eastern United States), *Physa wrighti*, and an individual of *Physa gyrina* (possibly *Physa wolfiana*) found at Hot Sulphur Springs, Colorado. Within the *Physa acuta*-like hot spring physids, *Physa cupreonitens* and *Physa spelunca* form genetically distinct and distant groups, which is indicative of a potentially effective barrier to gene flow between these hot springs and nearby populations of *Physa acuta*. Colonization of hot spring environments does not typically entail much sequence divergence from colder-water populations.

Key words: hot springs; cave; life history evolution; population; systematics

Species of the genus *Physa* Draparnaud, 1801 are more commonly found in heated waters than any other molluscan groups in North America and Europe (Clench 1926), so it is not surprising that there are a number of physids that were originally described from hot springs. Hot springs are localized, unique environments, many with a high sulphur content, providing their inhabitants with stable temperatures throughout the year. Springs, and in particular, cave springs, can have a significant impact on gene flow. For example, Gooch and Hetrick (1979) found that ecophenotypic gammarids in subterranean cave springs (and to some extent other spring populations of gammarids) have low heterozygosities compared to above-ground populations in first and second order streams. Low heterozygosities could be indicative of restricted or limited gene flow into these spring habitats. Given their constancy and persistence over long periods of geological time, it is possible that hot springs could be homes to relict species that have become endemic due to major climatic perturbations such as glaciation (Te and Clarke 1985).

Members of the *Physa acuta* group as well as members of the *Physa gyrina* group were first described from hot spring environments. Both groups of physids are common and widespread in North America. The *P. acuta* group is diagnosed by having penial morphology "c" (Te 1978), one non-glandular sheath and a penial gland, and has a worldwide distribution. There is evidence from laboratory breeding (Dillon *et al.* 2002) and molecular studies (A. R. Wethington, pers. obs.) that *Physa heterostropha* (Say, 1817) and *Physa integra* (Haldeman, 1842) should be synonymized under the name *Physa acuta* (Draparnaud, 1801). The *P. gyrina* group has penial morphology "b" (Te 1978), which includes a penial gland and two distinct sheaths, one glandular and one non-glandular. They are more constrained by geography and are excellent at persisting in harsh environments such as temporary ponds (Clampitt 1970).

Physids of both groups are able to withstand high temperatures as well as widely fluctuating temperatures. Individuals of *Physa gyrina* (Say, 1821) are well suited to live in the elevated temperatures of shallow waters (Clampitt 1970) as well as along a reach artificially heated to 35°C by effluent from a local manufacturing plant (Agersborg 1929). Individuals of *Physa anatina* (Lea, 1864) of the *Physa acuta* group can withstand temperatures as high as 40°C (Beames and Lindeborg 1968). Individuals of *Physa virgata* (Gould, 1855) of the *P. acuta* group did not alter their metabolic rate after living in artificially heated water for 54 generations (McMahon 1985), lending support to the hypothesis of Sandurathri and Holmes (1976) that some species of physids are pre-adapted to living in heated waters, which would allow them to invade hot spring habitats.

We focused on mitochondrial gene divergence between physids found in hot spring environments and nearby cold spring environments to compare the evolutionary histories of hot spring physids in open environments as well as in a cave. This study included six named and one unnamed species from hot or warm springs: *Physa aurea* (Lea, 1838), *Physa wrighti* (Te and Clarke, 1985), *Physa johnsoni* (Clench, 1926), and *Physa wolfiana* (Lea, 1869) of the *Physa gyrina* group; *Physa spelunca* (Turner and Clench, 1974), an unnamed physid from Mt. Princeton Hot Springs Colorado; and *Physa cupreonitens* (Cockerell, 1889) of the *Physa acuta* group (Table 1).

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Most descriptions of physid species rely heavily on shell morphology, including those first described from hot springs. Exceptions include *Physa spelunca* and *Physa wrighti*, in which detailed anatomical descriptions are available. Reliance on shell characters makes it hard to identify each putative species, especially outside its type locality, given the large degree of plasticity in shell characters. Shell shape can be influenced by the presence of predators (DeWitt 1995, DeWitt 1998, DeWitt *et al.* 1999, DeWitt *et al.* 2000, Langerhans and DeWitt 2002) as well as by environmental factors including temperature (Burnside 1998, Britton 2004).

According to the original descriptions, *Physa wrighti* and *Physa spelunca* have unusual anatomies that result from living in their environments. Individuals of *P. wrighti* share anatomical characters with three distinct groups (the *Physa acuta* group, the *Physa pomilia* group, and the *Physa gyrina* group) (Te and Clarke 1985). *Physa spelunca* was described as having no pigment (Turner and Clench 1974) but live specimens exhibit a wide range of color polymorphism that includes the albino form (Megan Porter, personal communication). Turner and Clench (1974) also describe *P. spelunca* as having reduced eyes and an increase in radular teeth size and protoconch size compared to other physids. These modifications could be adaptations to living in a cave with a constant, yet nutrient-poor environment, as Turner and Clench (1974) suggest.

Our main goal was to use sequence divergence as an estimate of how long a particular form of physid has been in a hot spring environment and to use inferences based on experiments on reproductive isolation with other physids to explore the validity of hot spring taxa. We argue that for physids first identified from hot springs to be considered good species, they should show amounts of mitochondrial sequence divergence similar to other species-level divergences in other pulmonates.

METHODS

Due to the difficulties that can arise from matching newly collected snails to original descriptions of the shell, we collected snails from the type locality of each species. This allowed us to compare sequence identity/divergence between them and other physids collected from a broad geographic area near each hot spring environment. Given the isolated nature of hot springs from colder water environments, physids originally described from a hot spring and subsequently collected from that spring should be the same species (see Table 1 for habitat descriptions).

Table 1. Shell lengths, shell widths, reported water temperatures, and current environmental information for each species of physid from hot springs.

Species	Site of original desciption	Shell length of holotype (mm)	Shell width of holotype (mm)	Reported water temperature (°C)	Number of springs mentioned	Human interference
Physa aurea	Hot spring at Bath, VA	12.7	7.62	21.1-26.7	one	tourists and industry
Physa wolfiana	Hot Springs, CO	7.62	4.8	37.7-43.3	one	man-made chlorinated pools from hot spring
Physa cupreonitens	Hot springs at Wellsville, CO	7.5	4.5	21.1-26.7	more than one	minimal, privately owned
Physa johnsoni	Middle spring of the Hot Sulphur Springs in Banff National Park, Alberta, Canada	7.5	5.2	~33.3	many	historically great, but now in park protected by Canadian law
Physa spelunca	Cave in Lower Kane Cave between 800-900 feet above Big Horn River, near Kane, WY	9.0	4.5	25.6	one	minimal, gate is locked and cave has toxic sulphuric gas
Physella wrighti	Alpha Stream, Liard Hot Springs Provincial Park, Alberta, Canada	5.3	2.8	~35	one	minimal, Provincial Park

Because *Physa wrighti* has been described as a basal member of the Physinae lineage, we have also included sampling from other major lineages within the group. If *P. wrighti* is indeed a relict left over from pre-glaciation events, then it should be distinctly different and basal to other physids, as predicted by Te and Clarke (1985). On the other hand, if *P. wrighti* is not genetically differentiated from surrounding physids, then it could have arisen from one or many recent migration events into the hot water habitat.

In addition to representatives from each hot spring and cave population, specimens from populations located near each hot spring and cave environment were included, as well as two representatives from the *Physa fontinalis* group (*Physa fontinalis* [Linnaeus, 1758] and *Physa jennessi* [Dall, 1919]) and the following physids from type locales: *Physa acuta* and *Physa virgata* of the *P. acuta* group, *Physa gyrina* of the *P. gyrina* group, *Physa zionis* (Pilsbry, 1926) which Te (1978) placed in its own genus (*Petrophysa* Te, 1978), and *Physa hendersoni* (Conrad, 1834) of the *Physa pomilia* group. For rooting purposes in the parsimony run, two planorbid species, *Biomphalaria obstructa* (Morelet, 1849) and *Planorbella trivolvis* (Say, 1817), and one lymnaid species, *Psuedosuccinea columella* (Say, 1817), were included as representatives of two freshwater basommatophoran families that are closely related to Physidae.

Shell and penial morphology of each individual was examined to aid in the initial placement of that individual into taxonomic groups. Specimens were preserved in 95% ethanol for DNA extraction. See Appendix 1 for taxonomic labels and locality data for each individual physid included in the present study.

DNA was extracted from one to three individuals per population using standard phenol chloroform procedures (Sambrook *et al.* 1989). Pieces of mtDNA from genomic DNA were copied and augmented via the Polymerase Chain Reaction using 16S primers (L2510 and H3080 = 16Sar-L and 16Sbr-H, Palumbi *et al.* 1991) for a 550 base pair segment and CO1 primers (LCO1490 and HCO2198, Folmer *et al.* 1994) for a 650 base pair segment, cleaned using standard procedures and then cycle-sequenced. The double-stranded PCR products were generated using 50-500 ng of template genomic DNA in 25 µl volumes (10 mM Tris, 50 mM KCL, 2.5 mM $MgCl_2$, 1 µM of each primer, 0.1 mM of each dNTP, 1.5 units Taq DNA polymerase; Fisher Scientific). The amplification regime began with a denaturation at 92°C for two minutes followed by 35 cycles of the following: denaturation at 92°C for 40 seconds, annealing at 52°C for 60 seconds (16S) or 50°C for 60 seconds (CO1), and extension at 68°C for 90 seconds. The amplified DNA was then concentrated using Millipore Ultrafree MC filters and provided the template for cycle sequencing using the ABI BigDye kit following manufacturer's instructions. The reactions were purified using Quiagen DyeEx spin columns and sequenced on an ABI3100 genetic analyzer.

The following physid sequences were obtained from genbank: *Physa johnsoni* (CApjomi, CApjolo, CApjoba, Capjoup, and CApjoca), *Physa gyrina* (CApgycb, CApgyml, CApgyfm, CApgycl and CApgyfly), *Physa wrighti* (CApwr323 and CApwr745), and *Physa* sp. (CApsp, probably *Physa jennessi* of the *Physa fontinalis* group) from Remigio *et al.* (2001).

The resulting sequences were aligned by eye directly for CO1 and by using the LSU rDNA secondary structure for 16S (Lydeard *et al.* 2000). Physids have a large number of base pairs in the coding loop portion of the three dimensional 16S rDNA subunit as compared to other pulmonate taxa. Within physids it was impossible to line up one taxon with another, so the loops were excluded from analysis of the 16S data set. The truncated 16S sequences were combined with the CO1 sequences for analysis.

Identical sequences were identified and removed from the phylogenetic analysis and the resulting data were analyzed using PAUP* (Swofford 2001). A parsimony heuristic search was performed with 50 random addition replicates to test relationships within and between the various physid groups using *Biomphalaria obstructa*, *Planorbella trivolvis*, and *Psuedosuccinea columella* to root the analysis. One hundred bootstrap replicates were performed with 1078 characters resampled at each replicate, optimality was set to parsimony, starting trees were obtained via stepwise addition, addition sequence was random each with ten replicates, and the branch swapping analysis was tree-bisection-reconnection (TBA). To obtain genetic distance measurements for the haplotype network, Modeltest (Posada and Crandall 1998) was employed to discover the best base pair substitution model for the physids only. For the combined analysis, the General Times Reversible model was modified with a gamma distribution parameter (G) and the estimated number of invariable sites (I) by hLRT in Modeltest version 3.06. The alpha level was set to 0.01. Base frequencies were as follows: A = 0.30180, C = 0.13850, G = 0.16990, and T = 0.38980. The assumed proportion of invariable sites was 0.3594. The Gamma distribution shape parameter was 0.7764. These settings were employed for 10,000 bootstrap replicates of a Neighbor-joining search using BioNJ method and maximum-likelihood distance measures.

The data were also run solely with CO1 to add *Physa wolfiana*. For the CO1 analysis, the General Times Reversible model was modified with a gamma distribution parameter (G) selected by hLRT in Modeltest version 3.06. The alpha level was set to 0.01. Base frequencies were as follows: A = 0.2808, C = 0.1389, G = 0.1454, and T = 0.4349. The proportion of invariable site (I) was none. The Gamma distribution shape parameter was 0.3612. This model was used in a neighbor-joining analysis with 10,000 replicates.

RESULTS

The best score for the heuristic search was a tree length of 1330, held by 80 trees, for the combined analysis (Fig. 1). The combined data set yielded 1078 total characters; 544 characters were constant, 145 characters were variable but uninformative, and 389 characters were variable and informative. The separate CO1 analysis retained 2122 trees, all of tree length 892, under the heuristic search criteria. The CO1 analysis yielded 658 total characters, 359 characters were constant, 67 characters were variable but uninformative, and 252 characters were variable and informative.

Penial morphology correlated with the resulting molecular phylogeny for Physidae in both analyses with four distinct groups represented: the *Physa acuta* group with bootstrap support of 99 (which included *Physa zionis*), the *Physa pomilia* group with bootstrap support of 100, the *Physa gyrina* group with bootstrap support of 100, and the *Physa fontinalis* group with bootstrap support of 97 for the combined analysis. Physids from hot springs either fell in the *P. acuta* group or the *P. gyrina* group and as such did not form a monophyletic group. In the combined analysis, each population from a hot spring for which more than one member was sampled represented a genetically distinct taxon within the *P. gyrina* group or the *P. acuta* group. The only exception was *Physa johnsoni* (Fig. 1), which was paraphyletic and included *P. gyrina* from Boyer River, Iowa (type locale of *P. gyrina*).

None of the physids from hot springs within the *Physa gyrina* group (*Physa johnsoni*, *Physa wrighti*, *Physa wolfiana*, and *Physa aurea*) were especially distant genetically from other geographically nearby *P. gyrina* taxa (ranging from 0-1.6% different). The largest genetic distances between a hot-spring physid and nearby *P. gyrina* was 0.012 substitutions per site for *P. aurea* of Virginia in the combined analysis (Fig. 2). This same pattern was seen in the CO1 analysis.

Physa cupreonitens was approximately 4.4% different from nearby coa021 (from Mount Princeton Hot Springs) and was 17.52% different from co43936 (also in Colorado but of another water drainage from the Arkansas River). *Physa spelunca* was about 10.45% different from wybhr2 collected just outside Kane cave in the Big Horn River (in the combined analysis). This gives support to there being an effective barrier to gene flow between these hot spring environments and nearby *Physa acuta* populations (Fig. 2). The same result was uncovered in the separate CO1 analysis.

DISCUSSION

Physids of the *Physa acuta* and *Physa gyrina* groups can apparently invade hot water environments; there is not a monophyletic hot spring physid group. This is not surprising considering that *P. acuta* and *P. gyrina* cannot successfully outcross (Wethington *et al.* 2000, Dillon *et al.* 2004). Physids living in hot springs of the *P. gyrina* group (*Physa johnsoni*, *Physa wrighti*, *Physa wolfiana*, and *Physa aurea*) are much more similar to each other genetically than are physids of the *P. acuta* group living in hot springs (*Physa cupreonitens*, *Physa spelunca*, and a physid from Mt. Princeton Hot Springs) (Figs. 1 and 2). *Physa wrighti* does not fall basal to the *P. gyrina* plus *P. acuta* plus *P. pomilia* groups as predicted by Te and Clarke (1985).

Within the *Physa acuta* group, *Physa cupreonitens* and *Physa spelunca* form monophyletic groups with 100% bootstrap support (Fig. 1). Although *P. cupreonitens* is most likely synonymous with *P. acuta*, *P. spelunca* is phylogenetically distinct and genetically distant enough that it could be a separate species, endemic to Lower Kane Cave. Its basal position within the *P. acuta* group indicates that *P. spelunca* has been separated from other *P. acuta* for nearly as long ago as the cave formed. According to A. S. Engel (pers. comm.), the cave probably formed by sulfuric acid speleogenesis, in which sulphuric acid cut into limestone, less than 10,000 years ago (post-glacial). This time estimate is based on the terrace development on the Bighorn River (A. S. Engel, pers. comm.). Given that physids growing in hot waters can reproduce continuously (Agersborg 1929), this could be sufficient time for the Lower Kane Cave population to have diverged significantly from other members of the *P. acuta* group. The physids in Lower Kane Cave are well protected and seem to thrive in their unique environment so the chance of losing *P. spelunca* seems minimal. Reproductive isolation has been discovered between *P. acuta* and one basal member of the *P. acuta* group, similarly distant genetically, uncovered in a recent molecular phylogeny (R. T. Dillon, pers. comm.).

Within the *Physa gyrina* group, *Physa wrighti*, *Physa aurea*, and the *Physa gyrina* of Coyner Springs, Virginia, USA, form distinct monophyletic groups (bootstrap support of 100, 86, and 67 respectively), but *Physa johnsoni* does not (Fig. 1). This same pattern was found in the CO1 analysis. This could, in part, be due to sampling, as there were only 2 individuals of *P. wrighti*, *P. aurea*, and *P. gyrina* from Coyner Springs represented in the analysis while there were 7 individuals of *P. johnsoni* represented. *Physa johnsoni*, with its small size and proportionately wide shell, is morphologically distinct from the local *P. gyrina*. Therefore, the identification of the cave spring population as *P. johnsoni* should be correct.

Hot sulphur water may cause physids to grow a smaller and more globose shell. This would be easy to test in the laboratory. Lepitzki *et al.* (2002) mention that they are rearing *Physa johnsoni* in the laboratory to re-introduce the snail to its former habitat as part of their recovery effort. The morphologies of the shells of *P. johnsoni* and *Physa cupreonitens* are very similar despite having long-separated ances-

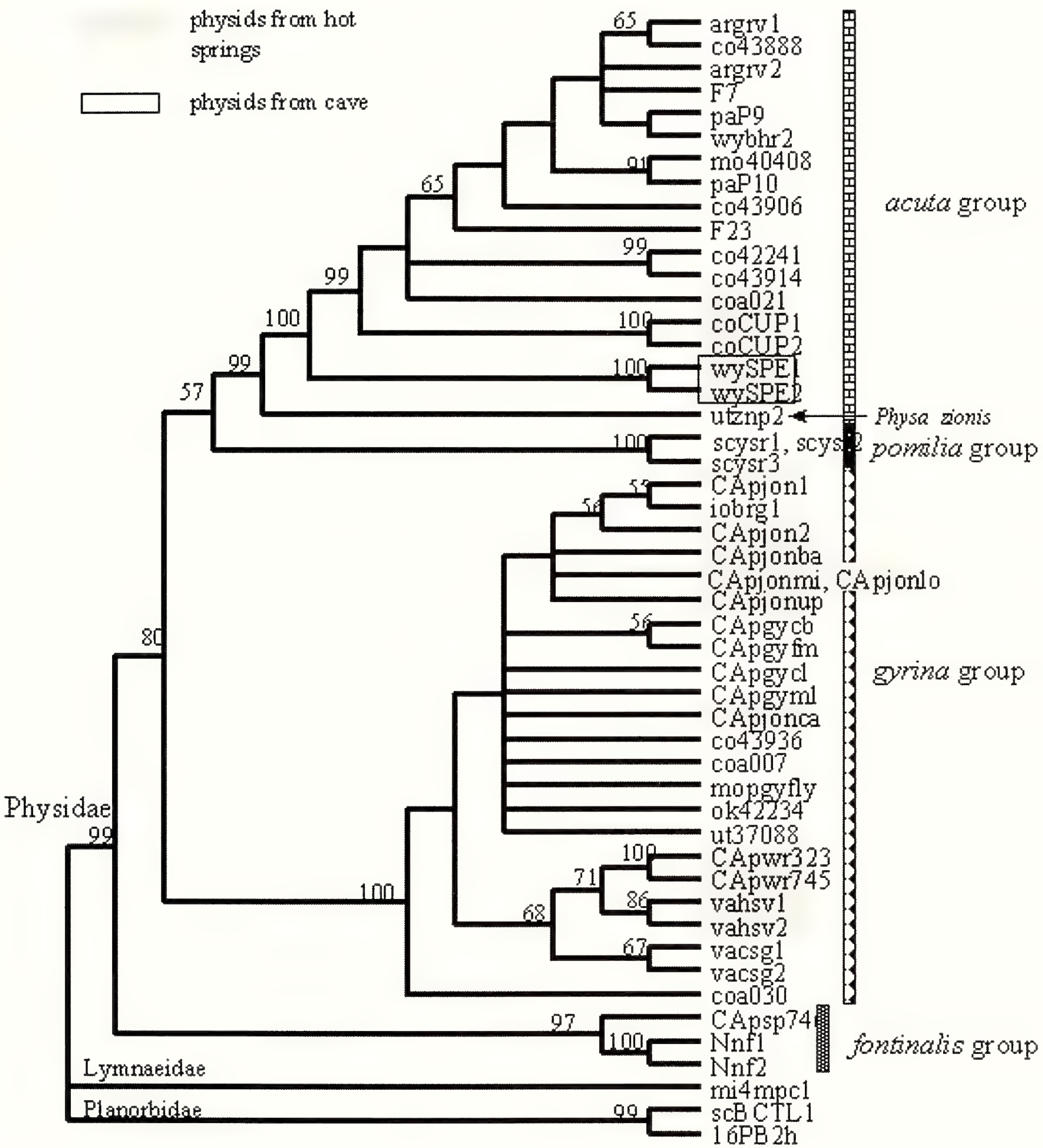
physids from hot springs
physids from cave
65
argrv1
co43888
argrv2
F7
paP9
wybhr2
91
mo40408
paP10
65
co43906
acuta group
F23
99
co42241
co43914
99
coa021
100
100
coCUP1
coCUP2
99
100
wySPE1
wySPE2
57
utznp2
Physa zionis
100
scysr1, scysr2
pomilia group
scysr3
53
CApjon1
56
iobrg1
CApjon2
CApjonba
CApjonmi, CApjonlo
CApjonup
56
CApgycb
CApgyfm
80
CApgycl
gyrina group
CApgyml
CApjonca
co43936
coa007
mopgyfly
ok42234
Physidae
99
ut37088
100
CApwr323
71
CApwr745
100
86
vahsv1
68
vahsv2
67
vacsg1
vacsg2
coa030
97
CApsp74
100
Nnf1
fontinalis group
Lymnaeidae
Nnf2
mi4mpc1
Planorbidae
99
scBCTL1
16PB2h

Figure 1. Parsimony tree of mtDNA 16S + CO1 with one hundred bootstrap replicates. The numbers above the nodes indicate bootstrap values. The shaded individuals are from hot spring habitats (*Physa spelunca* is from a cave) and are labeled as follows: CApjon = *Physa johnsoni*; CApwr = *Physa wrighti*; vahsv = *Physa aurea*; coa021 = Mt. Princeton Hot Springs, Colorado; coCUP = *Physa cupreonitens*, and wySPE = *P. spelunca*. See appendix for additional taxa and location information for each label.

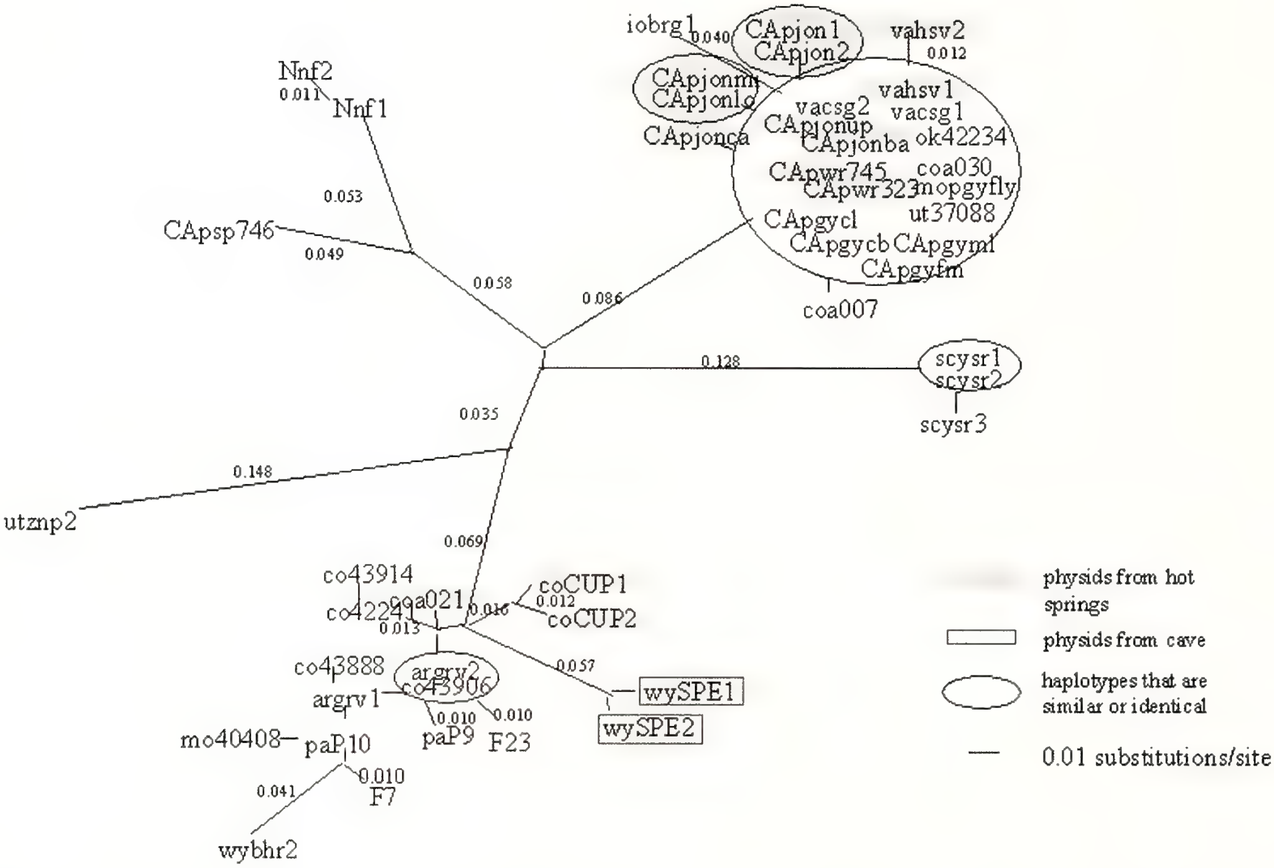
iobrg1
0.040
CApjon1
CApjon2
vahsv2
0.012
Nnf2
0.011
Nnf1
CApjonm
CApjonl
vahsv1
vacsg2
vacsg1
CApjonup
CApjonba
ok42234
CApjonca
CApwr745
coa030
CApwr323
mopgyfly
ut37088
CApgycl
CApgycb
CApgyml
CApgyfm
0.053
CApsp746
0.049
0.058
0.086
coa007
0.128
scysr1
scysr2
scysr3
0.035
0.148
utznp2
0.069
co43914
co42241
coa021
0.016
coCUP1
0.012
coCUP2
0.013
0.057
co43888
argrv2
co43906
argrv1
wySPE1
wySPE2
0.010
0.010
mo40408
paP10
paP9
F23
0.010
0.041
F7
wybhr2
physids from hot springs
physids from cave
haplotypes that are similar or identical
0.01 substitutions/site

Figure 2. Haplotype network showing the number of substitutions per site between each taxon or group of taxa. The values for nodes having a substitution per site greater than 0.01 are given. The individuals shaded are from hot spring habitats (*Physa spelunca* is from a cave) and are labeled as follows: CApjon = *Physa johnsoni*; CApwr = *Physa wrighti*; vahsv = *Physa aurea*; coa021 = Mt. Princeton Hot Springs, Colorado; coCUP = *Physa cupreonitens*, and wySPE = *P. spelunca*. See appendix for additional taxa and location information for each label.

tors. Britton (2004) found that *Physa virgata* from cold-water populations that were raised in warm waters (30-35°C) for five generations manifested differences in shell shape. The generation 5 individuals had larger spire angles and hence more globose shells compared to the source population living in cold water. Britton (2004) concludes that there is a genetic and environmental temperature-based component to shell shape in this species.

We assert that individuals of *Physa gyrina* invaded or were accidentally introduced into different hot springs environments and over a short period of time (as few as five generations) these populations evolved distinctive shells. The genetic distance between members of this *P. gyrina* group are generally not greater than 6% and as such are probably all representatives of the same species, *P. gyrina*. This is very low compared to the genetic distance found between pulmonate species in general (Thomaz *et al.* 1996, Ross 1999, Wade *et al.* 2000, Davison 2000, DeJong *et al.* 2001, A. R. Wethington, personal observation). Also, Dillon and Wethington (In press) have shown that *Physa aurea* can successfully outcross with *P. gyrina* to the F_2 generation. It is likely that all taxa of the *P. gyrina* group included in this study represent one biological species.

In conclusion, colonization of physids into hot spring environments does not seem to entail much sequence divergence, especially in members of the *Physa gyrina* group. The sequence divergence uncovered within the *Physa acuta* group could be due to sampling error or could reflect underlying ecological or physiological differences between the physids living in hot springs (particularly *Physa spelunca*) and the rest.

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Appendix 1. Identification label of each individual included in this study along with its accession numbers (where applicable), its classification, and where it was collected.

Identity	Acc. # 16S	Acc. # CO1	Genus	Subgenus	Species	Country	State/ Territory	County/ Region	Locality Data
argrv1	AY651170	AY651209	*Physa*	*Physella*	*virgata*	USA	Arizona		Gila River
argrv2	AY651171	AY651210	*Physa*	*Physella*	*virgata*	USA	Arizona		Gila River
Capjon1	AY651172	AY651211	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Middle Spring (type)
CApjon2	AY651173	AY651212	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Middle Spring (type)
CApgycb	AF346752	AF346740	*Physa*	*Physella*	*gyrina*	Canada	Alberta	Banff	C&B Marsh
CApgyml	AF346753	AF346741	*Physa*	*Physella*	*gyrina*	Canada	Alberta	Banff	Muleshoe Lake
CApgyfm	AF346754	AF346742	*Physa*	*Physella*	*gyrina*	Canada	Alberta	Banff	Five Mile Pond
CApgycl	AF346755	AF346743	*Physa*	*Physella*	*gyrina*	Canada	Alberta	Banff	Clear Spring
mtpgyfly	AF346756	AF346744	*Physa*	*Physella*	*gyrina*	USA	Montana	Flathead Lake	Flathead Lake; Yellow Bay
CApjomi	AF346747	AF346735	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Middle Spring (type)
CApjolo	AF346748	AF346736	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Lower Spring, Cave and Basin National Historic Site
CApjoba	AF346749	AF346737	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Basin Spring, Cave and Basin National Historic Site
CApjoup	AF346750	AF346738	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Upper Spring, Cave and Basin National Historic Site
CApjoca	AF346751	AF346739	*Physa*	*Physella*	*johnsoni*	Canada	Alberta	Banff	Cave Spring, Cave and Basin National Historic Site
CApsp	AF346758	AF346746	*Physa*	*Physa*	sp?	Canada	Alberta		
CApwr323	AF419322	AF419323	*Physa*	*Physella*	*wrighti*	Canada	Alberta	British Columbia	Laird Hot Springs
CApwr745	AF346757	AF346745	*Physa*	*Physella*	*wrighti*	Canada	Alberta	British Columbia	Laird Hot Springs
co42241	AY651174	AY651213	*Physa*	*Physella*	*acuta*	USA	Colorado	Mesa	creek south of Vega Reservoir, near campground
co43888	AY651175	AY651214	*Physa*	*Physella*	*anatina*	USA	Colorado	Yuma	Landsman Creek
co43906	AY651176	AY651215	*Physa*	*Physella*	*anatina*	USA	Colorado	Garfield	Elk Creek
co43914	AY651177	AY651216	*Physa*	*Physella*	*anatina*	USA	Colorado	Rio Blanco	Yellow Creek
co43936	AY651178	AY651217	*Physa*	*Physella*	*gyrina*	USA	Colorado	Garfield	Garfield Creek
coa006	AY651179	—	*Physa*	*Physella*	*wolfiana*	USA	Colorado	Grand	Colorado River near Hot Sulphur Springs
coa007	AY651180	AY651218	*Physa*	*Physella*	*gyrina*	USA	Colorado	Grand	western Tributary off Colorado River near Hot Sulphur Springs
coa021	AY651181	AY651219	*Physa*	*Physella*	*acuta*	USA	Colorado	Chaffe	Farmers Ditch, trib of Boulder Creek (Mt. Princeton Hot Springs)
coa030	AY651182	AY651220	*Physa*	*Physella*	*gyrina*	USA	Colorado		
coCUP1	AY651183	AY651221	*Physa*	*Physella*	*cupreonitens*	USA	Colorado	Fremont	Hot Springs in Wellesville, Colorado
coCUP2	AY651184	AY651222	*Physa*	*Physella*	*cupreonitens*	USA	Colorado	Fremont	Hot Springs in Wellesville, Colorado
F23	AY651185	AY651223	*Physa*	*Physella*	*acuta*	France	Saint-Martin de Londres		Rieutort Wadi, 25 km north of Montpellier, (near type)
F7	AY651186	AY651224	*Physa*	*Physella*	*acuta*	France	Saint-Martin de Londres		Rieutort Wadi, 25 km north of Montpellier, (near type)
iobrg1	AY651187	AY651225	*Physa*	*Physella*	*gyrina*	USA	Iowa		Boyer Rver, North of Council Bluffs (type)
mo40408	AY651188	AY651226	*Physa*	*Physella*	*acuta*	USA	Missouri	Reynolds	Hunter Hollow (off Black River)
Nnf1	AY651189	AY651227	*Physa*	*Physa*	*fontinalis*	Netherlands			Nooredemeek (Rykel de Bruyne's site: 133/481)
Nnf2	AY651190	AY651228	*Physa*	*Physa*	*fontinalis*	Netherlands			Nooredemeek (Rykel de Bruyne's site: 133/481)
ok42234	AY651191	AY651229	*Physa*	*Physella*	*gyrina*	USA	Colorado	Mesa	creek north Sunset Lake
paP10	AY651192	AY651230	*Physa*	*Physella*	*heterostropha*	USA	Pennsylvania	Philadelphia	Philadelphia; Schuylkill River at Fairmont Park
paP9	AY651193	AY651231	*Physa*	*Physella*	*heterostropha*	USA	Pennsylvania	Philadelphia	Philadelphia, Schuykill River at Fairmont Park
scysr1	AY651194	AY651232	*Physa*	*Physella*	*hendersoni*	USA	South Carolina	Hampton	South Carolina, Yamassee; Salkehatchie River off 17A, near 21
scysr2	AY651195	AY651233	*Physa*	*Physella*	*hendersoni*	USA	South Carolina	Hampton	South Carolina, Yamassee; Salkehatchie River off 17A, near 21
scysr3	AY651196	AY651234	*Physa*	*Physella*	*hendersoni*	USA	South Carolina	Hampton	South Carolina, Yemassee; Salkehatchie River off 17A, near 21
ut37088	AY651197	AY651235	*Physa*	*Physella*	*gyrina*	USA	Utah	Box Elder	(salt?) spring that empties into North Mud Flat of the Great Salt Lake

Appendix 1. (continued)

Identity	Acc. # 16S	Acc. # CO1	Genus	Subgenus	Species	Country	State/ Territory	County/ Region	Locality Data
utznp2	AY651198	AY651236	*Physa*	*Petrophysa*	*zionis*	USA	Utah	Zion National Park	Along rock face where seeping, The Narrows
vacsg1	AY651199	AY651237	*Physa*	*Physella*	*gyrina*	USA	Virginia		Coyner Springs
vacsg2	AY651200	AY651238	*Physa*	*Physella*	*gyrina*	USA	Virginia		Coyner Springs
vahsv1	AY651201	AY651239	*Physa*	*Physella*	*aurea*	USA	Virginia		Hot springs in Bath, Virginia
vahsv2	AY651202	AY651240	*Physa*	*Physella*	*aurea*	USA	Virginia		Hot springs in Bath, Virginia
wybhr2	AY651203	AY651241	*Physa*	*Physella*	*acuta*	USA	Wyoming		Big Horn River near Lower Kane Cave
wySPE1	AY651204	AY651242	*Physa*	*Physella*	*spelunca*	USA	Wyoming		Lower Kane Cave near Kane, Wyoming
wySPE2	AY651205	AY651243	*Physa*	*Physella*	*spelunca*	USA	Wyoming		Lower Kane Cave near Kane, Wyoming
mi4mpcl	AY651206	AY651244	*Psuedosuccinea*		*columella*	USA	Michigan		Four Mile Lake, Michigan
scbctl1	AY651207	AY651245	*Biomphalaria*		*obstructa*	USA	South Carolina	Charleston	Charles Towne Landing (see Dillon and Dutra-Clarke 1992) Malacological Review 25: 129-130
scpb2	AY651208	AY651246	*Planorbella*		*trivolvis*	USA	South Carolina	Charleston	off Bees Ferry Road

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RESEARCH NOTE

Method for mounting radulae for SEM using an adhesive tape desiccation chamber

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Abstract: A method to mount radulae for low-magnification SEM study is described. The wet radula was arranged over the adhesive side of a piece of adhesive tape. The addition of a few drops of liquid delayed desiccation, permitting the radula to be positioned. A piece of clear plastic film was added to form a desiccation chamber. Pinpricks around the radula allowed it to desiccate slowly. The desiccation chamber was then cut near the radula, and the radula and its adhesive base were mounted on an SEM stub. The method is simple and prepares radulae with little deformation.

Key words: radula, SEM, mounting, method, desiccation

The radula is often studied using a scanning electron microscope (SEM). Several methods are available to prepare and mount radulae. Among them, Solem (1972) and Bradner and Kay (1995) suggest mounting the radula directly on the SEM stub using a thin layer of adhesive (double-sided tape or contact cement). There are problems with this approach, however; it is difficult to reposition the radula once it touches the adhesive and the radular ribbon may curl and distort during desiccation, changing position or breaking. During the study of the *Cribrarula cribraria* (Linnaeus, 1758) species complex (Cypraeidae) (Moretzsohn 2003), I developed a simpler alternative that allowed ample time to manipulate the radula, minimized deformation due to desiccation, and prepared radulae suitable for SEM study at low magnification.

THE METHOD

I prepared a strip of adhesive tape with both ends looped backwards (adhesive side up) and attached it to a glass slide or piece of cardboard. I placed the radula flat with teeth up and radular ribbon down on the adhesive side of the adhesive tape (Fig. 1) in a drop or two of 70% ethanol. The radular ribbon could then be positioned. More liquid (25-70% ethanol) was added with a Pasteur pipette to delay desiccation while the radula was being arranged on the adhesive tape. The method worked best when the radula was wet but not excessively wet (in which case the radula would curl; Fig. 3). When the alcohol began to evaporate, the adhesive tape became sticky and the radula could be attached to the tape. It could be re-arranged if more alcohol was added.

I then covered the radula with non-adhesive clear plastic film (e.g. polyethylene or acetate) and pressed it against the adhesive tape all around the radula, thus forming a desiccation chamber with a wet radula inside. I used an insect pin to prick a few small holes around the radula, about 5-10 mm away from it (Fig. 2). The alcohol in the desiccation chamber evaporated slowly, depending on the temperature, humidity outside the chamber, and size of the holes. The clear film allowed me to monitor the radula as it desiccated. The radula was dry enough to be mounted on an aluminum SEM stub after 48 hours at room temperature. If the radula still looked wet or if there was condensation in the plastic chamber, I allowed it to dry longer. Extra pinpricks were necessary if desiccation was slow.

I prepared a clean aluminum SEM stub (Solem 1972), coating it with nail polish, double-sided adhesive tape, or other adhesive to bond the desiccation chamber to the stub. I cut the adhesive tape chamber close to the radula. Using a pair of fine tweezers, I removed the non-adhesive cover film and placed the adhesive base with the radula on the SEM stub (Figs. 4-5). I pressed the sides of the adhesive base close to the radula against the stub to ensure a good bond. It was important that the radula and its tape base were oriented so that the radula was uppermost. Use of colored adhesive tape helped prevent misalignment. I placed the SEM stub in an airtight container with fresh desiccant (silica gel or similar) as soon as the radulae were mounted to prevent the settling of dust particles (Hickman, pers. comm.) and to ensure complete desiccation before sputter-coating the specimen

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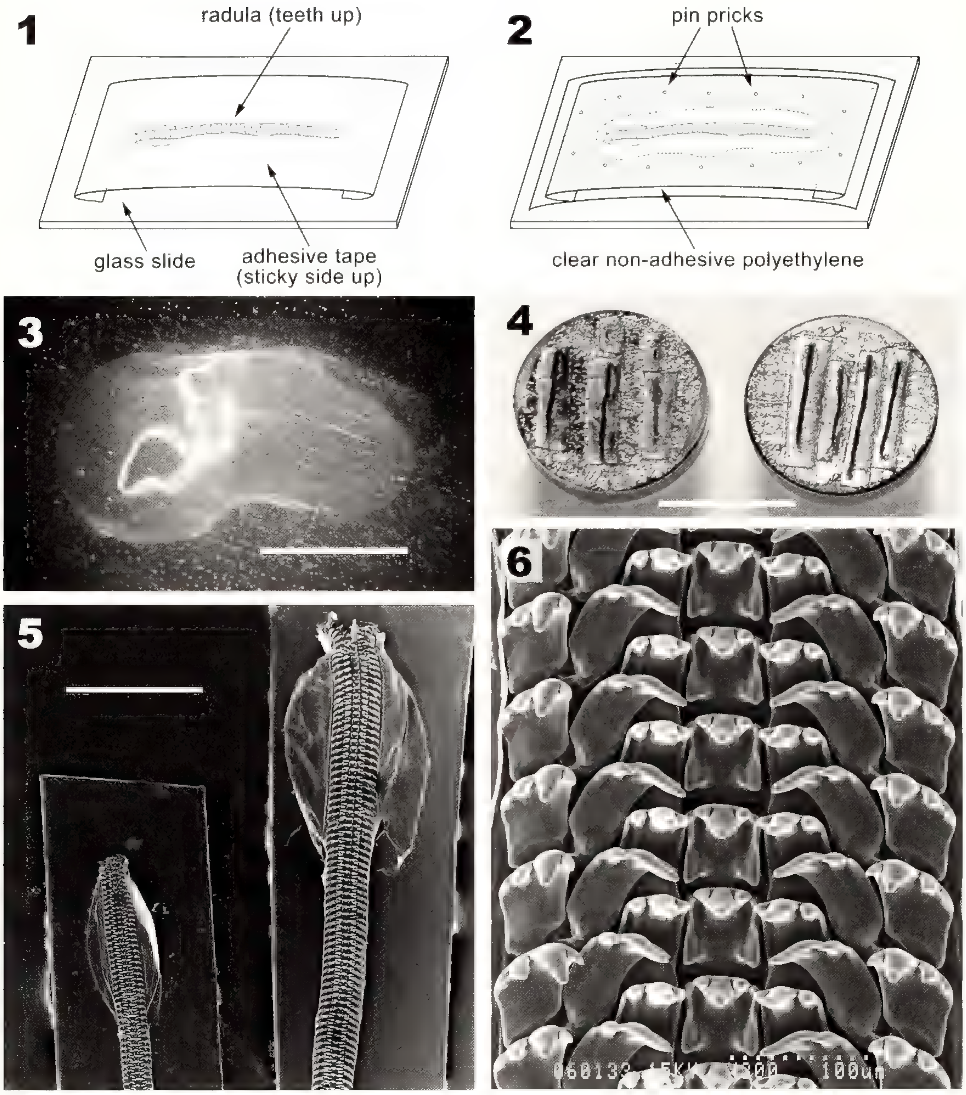

Figures 1-6. 1, Diagram of a wet radula arranged on the adhesive surface of adhesive tape. The radula can be positioned with insect pins or tweezers prior to covering it with a clear plastic film (Fig. 2). 2, Diagram of a desiccation chamber made with adhesive tape and clear non-adhesive polyethylene film for slow desiccation of radulae for SEM. Ethanol evaporates slowly through pinpricks made around the radula. 3, Photograph of a radula curling with excessive ethanol prior to being positioned in the desiccation chamber. Note the beads of adhesive on the surface of adhesive tape; scale = 10 mm. 4, Photograph of small radulae of cowries in the *Cribrarula cribraria* (Linnaeus, 1758) complex mounted on SEM stubs and coated with gold-palladium for SEM observation; scale = 10 mm. 5, Scanning electron micrograph showing parts of two radulae of *Cribrarula gaskoinii* (Reeve, 1846) prepared as described in text. Note the plastic base cut close to the radulae and mounted on SEM stubs; scale = 1500 mm. 6, Scanning electron micrograph of the radula of *Cribrarula gaskoinii* desiccated as described in the text, showing no significant distortion of the radula and individual teeth.

with gold-palladium (or equivalent) for observation with an SEM.

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Seed, R. 1980. Shell growth and form in the Bivalvia. *In*: D. C. Rhoads and R. A. Lutz, eds., *Skeletal Growth of Aquatic Organisms*, Plenum Press, New York. Pp. 23-67.

Yonge, C. M. and T. E. Thompson. 1976. *Living Marine Molluscs*. William Collins Son and Co., Ltd., London.

Dall, W. H. 1889. Reports on the results of dredging, under the supervision of Alexander Agassiz, in the Gulf of Mexico (1877-78) and in the Caribbean Sea (1879-80), by the United States Coast Survey Steamer "Blake," Lieutenant-Commander C. D. Sigsbee, U. S. N., and Commander J. R. Bartlett, U. S. N., commanding. Report on the Mollusca, Pt. 2: Gastropoda and Scaphopoda. *Bulletin of the Museum of Comparative Zoölogy* **18**: 1-492, pls. 10-40.

Orbigny, A, d'. 1835-46. *Voyage dans l'Amérique Méridionale (le Brésil, la République Orientale de l'Uruguay, la République Argentine, la Patagonie, la République du Chili, la République de Bolivia, la République du Pérou), exécuté pendant les années 1826, 1827, 1828, 1829, 1830, 1831, 1832 et 1833. Vol. 5, Part 3 (Mollusques)*. Bertrand, Paris. Dates of publication: pp. 1-48, [1835], pp. 49-184 [1836], pp. 185-376 [1837], pp. 377-408 [1840], pp. 409-488 [1841], pp. 489-758 + pls. 1-85 [1846].

Hurd, J. C. 1974. *Systematics and Zoogeography of the Unionacean Mollusks of the Coosa River Drainage of Alabama, Georgia and Tennessee*. Ph.D. Dissertation, Auburn University, Alabama.

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